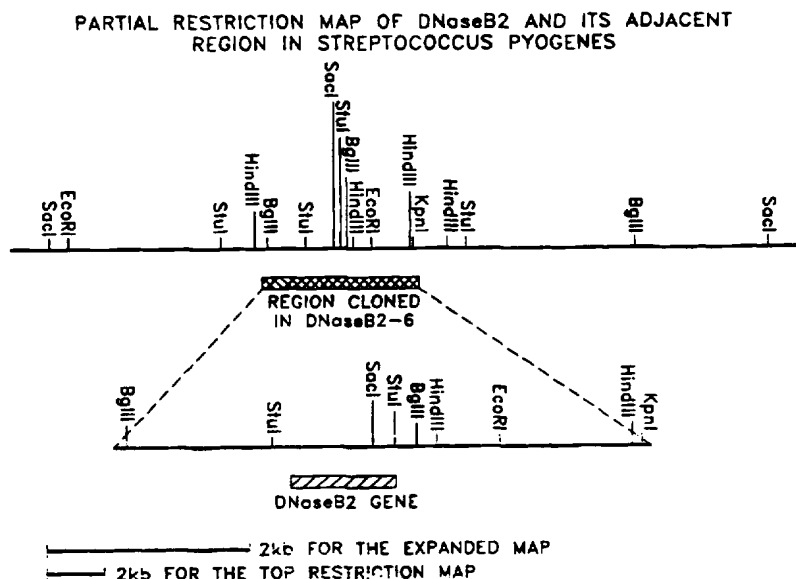




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(54) Title: RECOMBINANT DNase B DERIVED FROM STREPTOCOCCUS PYOGENES



(57) Abstract

The gene for *Streptococcus pyogenes* DNase B has been cloned and vectors incorporating the cloned DNA have been used to transform *Escherichia coli*, allowing efficient and rapid production of the DNase in *E. coli* without the necessity of growing large quantities of *S. pyogenes*. The enzyme can be produced with a leader peptide at its amino terminus. An improved method for the purification of naturally occurring *S. pyogenes* DNase B enzyme is also provided. The DNase B enzyme produced, either by purification of naturally occurring enzyme or by recombinant DNA techniques, can be used to generate antibodies and can also be used in immunochemical assays to detect the presence of anti-DNase B antibodies in serum as a marker of infection by *S. pyogenes*.

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RECOMBINANT DNASE B DERIVED FROM STREPTOCOCCUS PYOGENESBACKGROUND OF THE INVENTION

This invention is directed to recombinant DNase B derived from the pathogenic bacterium Streptococcus pyogenes, methods for its production, and methods for its use.

Despite advances in the prevention and treatment of bacterial infection, a number of bacterial pathogens remain serious problems in medical practice and continue to cause severe, even fatal disease. One of these pathogens is S. pyogenes. Among the diseases caused by S. pyogenes are streptococcal pharyngitis ("strep throat"), scarlet fever, and their suppurative complications, including cervical adenitis, otitis media, mastoiditis, peritonsillar abscesses, meningitis, pneumonitis, pneumonia, puerperal sepsis, cellulitis of the skin, impetigo, lymphangitis, erysipelas, acute glomerulonephritis, and rheumatic fever.

Such infections often occur in hospitals (nosocomial infection), particularly in patients whose normal immune system functioning is suppressed. The latter category includes patients with AIDS, patients taking immunosuppressive drugs for cancer or to prevent transplant rejection, and patients having poor circulation, e.g., patients with diabetes.

Because these diseases require rapid and effective treatment to eradicate the suppurative lesions and prevent sequelae caused by immunological reactions to persisting suppurative lesions, prompt diagnosis of the presence of S. pyogenes is essential in patients in whom such infections are

suspected. Failure to diagnose S. pyogenes promptly can greatly complicate treatment or even make it impossible.

5 Although detection methods for S. pyogenes are currently available, these methods have defects, particularly in clinical applications.

10 Among the methods of detection of S. pyogenes is the detection of the presence of antibodies against DNase B, a DNA-degrading enzyme produced by S. pyogenes. This enzyme, which is excreted from S. pyogenes during infection, initiates development of substantial titers of antibody in patients who go on to develop acute rheumatic fever and acute glomerulonephritis.

15 Although other serum-based diagnostic tests for these rheumatic fever and glomerulonephritis are available, including the detection of antibodies to streptolysin O, and to hyaluronidase, assays for anti-DNase B antibodies offer certain advantages, because DNase B is found among nearly all strains of
20 group A beta-hemolytic streptococci, and because high DNase B titers are found in patients with infections of the skin and pharynx.

25 Although a number of commercially-available tests exist for the assay of anti-DNase B antibody, these tests have defects. As indicated above, an improved test is greatly needed.

30 The commercially-available tests fall into three categories: (1) a DNase B inhibition-based assay using the ability of the antibody to inhibit enzymatic activity; (2) a latex agglutination assay for antibody against a variety of S. pyogenes antigens; and (3) a turbidimetric inhibition assay. ELISA assays have also been used in the research laboratory, but, as detailed below, they have not yet proven suitable for routine
35 clinical application.

 The DNase B inhibition assay is very slow, and

typically requires about 4-8 hours to perform. Thus, in situations in which confirmation of anti-DNase B antibody is required rapidly so the treatment can be started as soon as possible should the presence of S. pyogenes be confirmed, the enzyme inhibition assay is not particularly useful.

The latex agglutination assay is designed to detect antibodies to five S. pyogenes antigens. However, test results indicate poor agreement between the latex agglutination assay and a specific anti-DNase B tests. In one study, G.C. Klein & W. L. Jones, "Comparison of the Streptozyme Test with the Antistreptolysin O, Antideoxyribonuclease B, and Antihyaluronidase Tests," App. Microbiol. 21:257-259 (1971), 12 out of 80 patients that tested negatively in the latex agglutination assay were, in fact, positive for anti-DNase B antibody. This high level of false negative results means that the test is undesirable for clinical use.

The turbidimetric inhibition assay depends on the inhibition of agglutination of latex particles coated with anti-DNase B antibody by a limiting quantity of a crude preparation of DNase B in the presence of serum containing anti-DNase B antibody, which competes for the antibody on the latex particles. This assay, which is described in U.S. Patent No. 5,055,395, incorporated herein by this reference, is relatively insensitive. Therefore, it is not suitable for use in the early stages of S. pyogenes infection, and it is precisely this period when accurate detection of the anti-DNase B antibody is most important. Additionally, the reagents used in the turbidimetric inhibition assay are difficult to manufacture.

ELISA-based assays for anti-DNase B antibody are reported in M.A. Gerber et al., "Enzyme-Linked Immunosorbent Assay of Antibodies in Human Sera to Streptococcal DNase B," J. Lab. Clin. Med. 95:258-265 (1980). Although these assays have proven effective as research tools, their scale-up for commercial use, particularly in clinical practice, has been impractical.

This is because such scale-up would require production and purification of the DNase B enzyme of Streptococcus pyogenes, which is, as detailed above, a serious pathogen. Not only would extremely costly containment methods be required for growth of this pathogenic bacterium in the quantity required to produce sufficient enzyme for commercialization of the ELISA assay, the media required for the growth of S. pyogenes is very complex and expensive. These concerns have seriously hampered development of a commercial version of the ELISA assay for anti-DNase B antibody.

Therefore, there exists a need for an improved, rapid, and specific assay for anti-DNase B antibody. Preferably, such an assay would be usable by a physician in his office and would require minimal equipment. This is because patients with diseases such as strep throat or scarlet fever typically see their family physician prior to hospitalization, and accurate diagnosis of S. pyogenes infection at that point would be preferable to a subsequent diagnosis made only when the patient has been hospitalized.

The development of such an improved assay is dependent on the availability of large quantities of DNase B enzyme itself. Therefore, there is also a need for a method for the production of S. pyogenes DNase B enzyme using a procedure that can be scaled up to produce commercial quantities of the enzyme without requiring complex, unwieldy, and expensive containment measures.

SUMMARY

We have cloned and expressed the gene for S. pyogenes DNase B in Escherichia coli, allowing convenient and efficient production of the DNase B enzyme without requiring the growth of S. pyogenes.

This cloning procedure results in substantially

purified DNA encoding an amino acid sequence selected from the group consisting of the amino acid sequence of: (i) Streptococcus pyogenes DNase B enzyme as shown in Figure 4, below, which enzyme includes at its amino terminus an arginine (R) residue derived from a leader peptide and absent in the natural DNase B enzyme; and (ii) a sequence encoding a functional equivalent of S. pyogenes DNase B enzyme, optionally including at least one residue of the leader peptide. The DNA is substantially free of DNA other than DNA encoding the S. pyogenes DNase B sequence of Figure 4, DNA encoding a functional equivalent of S. pyogenes DNase B enzyme, and DNA encoding the leader peptide.

Preferably, the DNA further comprises a DNA sequence coding for a leader peptide fused to the amino terminus of S. pyogenes DNase B enzyme.

Most preferably, the DNA cloned is the DNA whose sequence is given in Figure 3, including the DNA coding for the entire amino acid sequence of S. pyogenes DNase B enzyme and the leader peptide.

Another aspect of the invention is expression vectors for Streptococcus pyogenes DNase B enzyme comprising the DNA sequences described above operatively linked to at least one control sequence compatible with a suitable bacterial host cell. Preferably, the expression vector is a plasmid vector. Typically, the DNA encoding the Streptococcus pyogenes DNase B enzyme is linked to at least one sequence from bacteriophage λ .

Another aspect of the invention is a bacterial host cell transformed, transfected, or infected with an expression vector according to the present invention in a manner allowing the transformed bacterial host cell to express the Streptococcus pyogenes DNase B encoded by the DNA incorporated within the expression vector in a detectable quantity. The expressed S. pyogenes DNase B can be either excreted or not excreted by the

whole cell producing the enzyme, and can be in a soluble or an insoluble form.

5 Another aspect of the invention is substantially purified S. pyogenes DNase B enzyme comprising a protein having the amino acid sequence of Figure 4, or having an amino acid sequence substantially similar thereto.

10 Yet another aspect of the invention is a process for producing substantially purified Streptococcus pyogenes DNase B enzyme comprising:

- (1) culturing the bacterial host cell transformed with an expression vector according to the present invention;
- (2) using the cultured bacterial host cell to express
15 the DNase B enzyme; and
- (3) purifying the enzyme from the cultured bacterial host cell.

20 Another aspect of the invention is Streptococcus pyogenes DNase B enzyme fused at its amino terminus with a leader peptide, the leader peptide having the sequence M-N-L-L-G-S-R-R-V-F-S-K-K-C-R-L-V-K-F-S-M-V-A-L-V-S-A-T-M-A-V-T-T-V-T-L-E-N-T-A-L-A-R (SEQ ID NO: 1).

25 Yet another aspect of the invention is a mutant of the protein whose amino acid sequence is shown in Figure 4. In the mutant, at least one of the following mutations occurs:

- (1) a deletion of one or more amino acids from the sequence of Figure 4;
- 30 (2) an insertion of one or more naturally-occurring L-amino acids into the sequence of Figure 4; and
- (3) replacement of at least one of the amino acids of Figure 4 with an alternative naturally occurring L-amino acid. The resulting mutant has reduced or increased DNase B activity
35 or another altered property. In one preferred alternative, the mutant substantially retains the antigenic reactivity of natural S. pyogenes DNase B enzyme.

Yet another aspect of the invention is the translational or transcriptional fusion of all or part of the S. pyogenes DNase B gene or protein to another gene or protein, with the resulting genetic construction having some altered property. These properties can include: (1) high level RNA expression; (2) high level protein expression; (3) a second functional enzyme, receptor, or other active protein in the fusion; (4) the fusion of the DNase B to an affinity ligand; (5) the production of a higher molecular weight protein; and (6) increased immunoreactivity.

Still another aspect of the invention is substantially purified natural Streptococcus pyogenes DNase B enzyme substantially free of proteins other than Streptococcus DNase B enzyme and Streptococcus DNase B enzyme fused at its amino terminus with a leader peptide. The substantially purified protein is substantially free of mitogenic activity. The substantially purified enzyme can be further purified into two fractions, Fraction I and Fraction II, depending on isoelectric point (pI). Each fraction can be purified into a preparation substantially free of the other fraction.

A process according to the invention for preparing substantially purified natural S. pyogenes DNase B enzyme can comprise:

(1) absorption to and elution from diethylaminoethyl cellulose to produce a first eluate;

(2) chromatography of the first eluate on phenyl agarose to produce a second eluate;

(3) chromatography of the second eluate on heparin agarose to produce a third eluate; and

(4) chromatofocusing of the third eluate to produce substantially purified DNase B enzyme. Preferably, the process further comprises purification of the substantially purified DNase B by reverse-phase high-pressure liquid chromatography. The separation of Fractions I and II occurs at the chromatofocusing step as a consequence of the differing pI's of

the enzymes of the two fractions.

5 Yet another aspect of the invention is a single-stranded nucleic acid probe hybridizing with at least about 17 nucleotides of the DNA sequence coding for the amino-terminal 23 amino acids of the Streptococcus pyogenes DNase B enzyme, not including any portion of the leader sequence thereof, with no greater than about a 30% mismatch.

10 A further aspect of the present invention includes portions of the DNA sequence of sufficient size and specificity to serve as primer sites for amplification reactions such as polymerase chain reaction (PCR), ligase chain reaction (LCR), RCR, or other DNA amplification reactions. The same portions of
15 the DNA sequence of S. pyogenes B can also serve as specific probes for detection of homologous sequences without DNA amplification.

20 The substantially purified S. pyogenes DNase B can be used to generate antibodies specifically binding the DNase B by techniques well known in the art. The antibodies can be either polyclonal or monoclonal.

25 Another aspect of the invention is a method for detecting and/or determining anti-Streptococcus pyogenes DNase B antibody in a test sample. The method comprises the steps of:

(1) providing a test sample suspected of containing anti-Streptococcus pyogenes DNase B antibody;

30 (2) adding a quantity of Streptococcus pyogenes DNase B enzyme according to the present invention to the test sample, the quantity being sufficient to produce a detectable level of enzymatic activity in the absence of inhibition of the enzymatic activity by anti-DNase B antibody in the test sample; and

35 (3) determining a level of activity of DNase B enzyme in the test sample by performing an enzyme assay to detect and/or determine the anti-Streptococcus pyogenes antibody in the test sample.

An alternative method for detecting anti-DNase B antibody comprises the steps of:

(1) binding Streptococcus pyogenes DNase B enzyme according to the present invention to a solid support such as latex particles;

(2) reacting a test sample suspected of containing anti-Streptococcus pyogenes DNase B antibody with the Streptococcus pyogenes DNase B enzyme bound to the solid support to bind the antibody to the enzyme and thus to the solid support; and

(3) detecting the antibody bound to the solid support to detect and/or determine the antibody in the test sample.

This approach can be used for nephelometric, turbidimetric, agglutination, or ELISA methods of quantitation.

An alternative method for detecting S. pyogenes DNase B antibody comprises:

(1) preparing a buffered solution of DNase B; (2) reacting the buffered DNase B solution with a test sample suspected of containing anti-S. pyogenes DNase B antibody; and (3) detecting a reaction between the DNase B and the anti-DNase B antibody by observing and/or measuring a change in light absorption and/or light scattering in the solution.

Another alternative method for detecting anti-DNase B antibody is capillary electrophoresis.

Because the cloned sequence includes a promoter associated with the S. pyogenes DNase B, gene, yet another aspect of the invention is a method of using the promoter originally associated with the S. pyogenes DNase B gene to express a protein other than DNase B. This method comprises:

(1) separating the promoter originally associated with the S. pyogenes DNase B gene from the S. pyogenes DNase B gene;

(2) operatively linking the promoter with a structural gene for a S. pyogenes protein other than the gene for DNase B;

and

(3) expressing the protein encoded by the structural gene.

5 The protein can be expressed in S. pyogenes, or in a prokaryote other than S. pyogenes.

10 Another aspect of the invention is a substantially purified promoter sequence derived from the promoter sequence originally associated with S. pyogenes DNase B including therein a start site for transcription and sites homologous to the consensus -10 and -35 sites of bacterial promoters.

15 Yet another aspect of the present invention is the use of the leader peptide of DNase B with the sequence M-N-L-L-G-S-R-R-V-F-S-K-K-C-R-L-V-K-F-S-M-V-A-L-V-S-A-T-M-A-V-T-T-V-T-L-E-N-T-A-L-A-R (SEQ ID NO: 1) to express a protein in a prokaryote. This aspect derives from the finding that when the entire cloned DNase B DNA segment, including the leader peptide, is expressed
20 in Escherichia coli, the protein is excreted into the culture medium. A process for using the leader peptide to express a protein in a prokaryote comprises:

(1) fusing the DNA coding for the protein to DNA coding for the leader peptide so that the fused DNA codes for a
25 recombinant protein with a single reading frame with the leader peptide being at the amino-terminus of the protein;

(2) introducing the fused DNA into the prokaryote; and

(3) expressing the fused DNA in the prokaryote so that the recombinant protein is produced in a recoverable quantity.

30

The prokaryote can be E. coli or a gram-positive bacterium such as a Staphylococcus, Streptococcus, or Streptomyces species.

35

Another aspect of the present invention is a method for immunizing a mammal against infection with S. pyogenes comprising administering a quantity of purified S. pyogenes DNase B enzyme

according to the present invention to the mammal sufficient to stimulate production of antibodies specific for S. pyogenes DNase B.

5 Yet another aspect of the present invention is a method for treating cystic fibrosis in a patient with cystic fibrosis. The method comprises:

(1) generating an aerosol of a purified enzymatically active DNase B enzyme according to the present invention; and

10 (2) administering the aerosol to a patient with cystic fibrosis in a quantity sufficient to reduce lung fluid viscosity in the patient.

15 BRIEF DESCRIPTION OF THE DRAWINGS

 These and other features, aspects and advantages of the present invention will become better understood with reference to the following description, appended claims, and the accompanying drawings where:

 Figure 1 shows a partial restriction map of the region containing cloned DNase B, indicating the region of chimeric DNA in the clone and the location of the gene for DNase B;

25 Figure 2 shows the locations of subclones of the cloned DNA of Figure 1 and an indication of nuclease activity produced by the subclones;

 Figure 3 shows the DNA sequence of the clone whose partial restriction map is shown in Figure 1;

30 Figure 4 shows the amino acid sequence of the recombinant DNase B protein derived from the DNA sequence of Figure 3, with the amino terminus determined as the result of sequencing of purified recombinant DNase B;

35 Figure 5 shows the DNA sequence of a construction to fuse the bacteriophage λ promoter to the DNA coding for the DNase B sequence, together with the primers used for PCR in forming the construction;

Figure 6 is a graph depicting the inactivation of recombinant DNase B by human anti-DNase B serum;

Figure 7 shows the DNA sequence upstream of the open reading frame in the cloned DNA and the consensus sequence of an E. coli promoter;

Figure 8 is a correlation curve indicating the agreement between determination of anti-DNase B antibody in human serum using recombinant DNase B enzyme and using commercially available DNase B enzyme isolated from S. pyogenes; and

Figure 9 is a graph indicating the essential absence of mitogenic activity from both recombinant DNase B and purified preparations of naturally occurring DNase B.

Figure 10 shows the DNA sequence of a genetic construction that is a component of a vector expressing DNase B in Escherichia coli that is processed identically to the native S. pyogenes DNase B (SEQ ID NO: 14), as well as the resulting protein sequence (SEQ ID NO: 15).

DESCRIPTION

In order to meet the need for a commercially useable source of Streptococcus pyogenes DNase B enzyme, we have cloned the gene for DNase B from S. pyogenes genomic DNA into Escherichia coli. Despite the considerable evolutionary difference between S. pyogenes and E. coli, as indicated by the considerable divergence in the sequence of the 18 S ribosomal RNAs of the two species, as well as the substantial difference in morphology and other taxonomic characteristics (E. coli is a gram-negative bacillus while S. pyogenes is a gram-positive coccus), we have achieved such a high level of expression in E. coli of the cloned gene and of activity of the expressed protein that screening could be performed by an enzymatic assay dependent on the activity of the expressed protein.

35

I. CLONING AND EXPRESSION OF STREPTOCOCCUS DNASE B GENE IN E. COLI

The cloning and expression of the Streptococcus pyogenes DNase B gene in E. coli requires the following steps, which are optimized carefully to achieve cloning of the intact gene in a form in which active enzyme is expressed from the gene:

- 5 (1) isolation of genomic DNA;
- (2) preparation of genomic DNA fragments for DNA cloning;
- (3) incorporation of DNA fragments into cloning vectors;
- 10 (4) infection of bacteria and selection; and
- (5) expression and screening;
- (6) characterization of the clone and DNA sequencing.

A. Isolation of Genomic DNA

15 Genomic DNA is preferably isolated from S. pyogenes under conditions minimizing activity of endogenous nucleases as well as other factors that can degrade or denature DNA. This requires cell lysis and degradation of protein. A preferable method for lysing cells is incubation with the proteolytic enzyme achromopeptidase at 65°C, followed by incubation with the chaotropic detergent sodium dodecyl sulfate (SDS). This procedure is most preferably carried out in the presence of a chelating agent such as EDTA. Alternatively, other proteases such as pronase and proteinase K can be used to lyse the cells.
20 Other lysis procedures are known in the art. (S. Horinouchi et al., "A New Isolation Method of Plasmid Deoxyribonucleic Acid from Staphylococcus aureus Using a Lytic Enzyme of Achromobacter lyticus," Agric. Biol. Chem. 41:2487-2489 (1977)).

30 Preferably, DNA is then extracted with phenol or phenol-chloroform and the extracted DNA is precipitated with ethanol. A suitable extraction sequence is two extractions with an equal volume of phenol, followed by one extraction with a 1:1 mixture of phenol/chloroform (Example 1). The extraction buffer preferably contains a chelating agent such as EDTA to minimize
35 nuclease activity. Such techniques are well known and are described, for example in D. M. Wallace, "Large- and Small-Scale

Phenol Extractions," Meth. Enzymol. 152:33-40 (1987) and in D. M. Wallace, "Precipitation of Nucleic Acid," Meth. Enzymol. 152:41-48 (1987).

5 A suitable source of DNA is strain ATCC No. 14289 of S. pyogenes, also known as C203S, a non-M containing variant of strain C203. However, similar techniques could be used for other strains of S. pyogenes that contain the gene for DNase B.

10 Preferably, the isolated DNA is treated with RNase A after extraction and ethanol precipitation, then further purified in a cesium chloride gradient.

B. Preparation of DNA Fragments for Cloning

15

The isolated genomic DNA is preferably fragmented before cloning. Most preferably, fragmentation is performed by passing the DNA through a syringe needle, most preferably a 25-gauge syringe needle, about 300 times. This results in sheared DNA having an average size of approximately 6-8 kb.

In a less preferred alternative, partial digestion with a restriction endonuclease can be used, such as Sau 3A or Mbo I. This is described, for example, in A.-M. Frischauf, "Digestion of DNA: Size Fractionation," Meth. Enzymol. 152:183-189 (1987), incorporated herein by this reference.

25

C. Incorporation of DNA Fragments Into Cloning Vectors

30

The next step is the incorporation of the DNA fragments into the appropriate cloning vector. Such a cloning vector typically comprises the DNA sequence coding for S. pyogenes DNase B operatively linked to at least one control sequence compatible with a suitable bacterial host cell. Such control sequences include operators and promoters. Suitable promoters include bacteriophage λ p_L promoter, a hybrid trp-lac promoter, and

35

bacteriophage T7 promoter. The cloning vector preferably also comprises a suitable ribosome-binding site for expression. A preferred cloning vector is λ gt11 (R. A. Young and R. W. Davis, Proc. Natl. Acad. Sci. USA 80:1194 (1983), which allows
5 expression controlled by a lac promoter incorporated into the vector and operatively linked to the cloned DNA. Other suitable cloning vectors are well-known in the art and are described, for example, in J. Sambrook et al., "Molecular Cloning: A Laboratory Manual" (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring
10 Harbor, New York, 1989), vol. 3, ch. 17, entitled "Expression of Cloned Genes in Escherichia coli", and incorporated herein by this reference. For phage λ gt11, the DNA is inserted into an Eco RI site. For such cloning the sheared DNA is preferably repaired using E. coli ligase and then T4 DNA polymerase, followed by the
15 addition of Eco RI linkers. These Eco RI-terminated fragments can be ligated to λ gt11 arms after digestion with Eco RI restriction endonuclease. Preferably, during this digestion procedure, the internal Eco RI sites are blocked by the use of Eco RI methylase, as the restriction endonuclease does not digest
20 DNA methylated at the adenine residues in the recognition site by the methylase.

After completion of the ligation reaction, the DNA is packaged into bacteriophage λ heads in vitro using a mixture of
25 extracts prepared from bacteria infected with bacteriophage λ mutants in genes required for assembly of phage particles. Packaging procedures are well-known in the art and are described, e.g. in Sambrook et al., supra, vol. 1, pp. 2.95-2.108.

30 D. Infection of Bacteria and Selection

The phage particles assembled by in vitro packaging are used to infect susceptible E. coli bacteria. A particularly preferred strain of bacterial host cells is Y1090 (-pMC9), that is, lacking the pMC9 plasmid. A suitable method is to overlay
35 the plaques with a top agar overlay of DNase test agar (Difco, Detroit, Michigan) containing 0.01% toluidine blue O as a color indicator. This allows detection of plaques expressing the DNase

B gene.

5 The unexpectedly high level of expression of the DNase B gene in this system allowed direct detection of positive clones by direct detection of the resulting enzymatic activity, without a need for immunological screening, which is commonly required for the detection of cloned gene products.

10 A process for producing substantially purified Streptococcus pyogenes DNase B enzyme using transfected host cells can comprise:

(a) culturing a bacterial host cell transformed with a suitable expression vector which can be a bacteriophage λ derivative;

15 (b) using the cultured transformed bacterial host cell to express the DNase B enzyme; and

(c) purifying the enzyme from the cultured transformed bacterial host cell.

20 E. Characterization of the Clone and DNA Sequencing

The λ gt11 phage containing the S. pyogenes DNase B gene (designated 2-6) was isolated and DNA was prepared from the phage. This clone was analyzed by restriction analysis and the results are shown in Figure 1. Analysis of Eco RI and Eco RI/Sac I subclones for the presence of nuclease activity indicates that part of the DNase B gene was located within the internal Sac I to the Eco RI region, as shown in Figure 2.

30 Sequencing of the cloned DNA can be performed using standard techniques, e.g. the Sanger dideoxynucleotide chain termination method. Sequence analysis can be initiated by priming synthesis within the λ gt11 phage across the suspected region of DNase activity. Results of such sequencing are shown in Figure 3.

35 The cloned DNA whose sequence is shown in Figure 3 incorporates a lengthy open reading frame (ORF). The amino acid

sequence derived from translation of this ORF is shown in Figures 3 and 4. The amino acid sequence of the 5'-terminal portion of this ORF starting at amino acid 44 (Gln) is consistent with the amino acid sequence derived by sequencing purified naturally occurring S. pyogenes DNase B (Section IV).

Accordingly, the invention encompasses substantially purified DNA comprising DNA encoding an amino acid sequence selected from the group consisting of the amino acid sequence of: (i) Streptococcus pyogenes DNase B enzyme as shown in Figure 4; and (ii) a sequence encoding a functional equivalent of S. pyogenes DNase B enzyme. The DNA is substantially free of DNA that does not encode the amino acid sequence of Figure 4 or a functional equivalent of S. pyogenes DNase B enzyme except for a leader peptide fused to the amino terminus of S. pyogenes DNase B enzyme. As discussed below, the translation product produced from the open reading frame includes a leader peptide.

In this context, the term "functional equivalent" refers to a protein possessing DNase activity detectable in the generally used assays for S. pyogenes DNase B and cross-reacting to at least a detectable extent with antibodies against substantially purified DNase B. The term "functional equivalent" includes, but is not limited to, proteins whose sequence differs from the sequence of Figure 4 by one or more conservative amino acid substitutions. Such conservative amino acid substitutions include, but are not limited to, substituting any of isoleucine (I), valine (V), and leucine (L) for any other of these amino acids; aspartic acid (D) for glutamic acid (E) and vice versa; glutamine (Q) for asparagine (N) and vice versa; and serine (S) for threonine (T) and vice versa. The above-mentioned substitutions are not the only amino acid substitutions that can be considered "conservative." Other substitutions can also be considered conservative, depending on the environment of the particular amino acid. For example, glycine (G) and alanine (A) can frequently be interchangeable, as can be alanine and valine (V). Methionine (M), which is relatively hydrophobic, can frequently be interchanged with leucine and isoleucine, and

sometimes with valine. Lysine (K) and arginine (R) are frequently interchangeable in locations in which the significant feature of the amino acid residue is its charge and the differing pK's of these two amino acid residues are not significant. Still other changes can be considered "conservative" in particular environments.

Also within the scope of the present invention are DNA sequences comprising a portion of the sequence of Figure 3 of sufficient size and specificity to serve as a reactant in a reaction requiring specific base hybridization. Such a DNA sequence can be a primer for an amplification reaction such as the polymerase chain reaction (PCR), ligase chain reaction (LCR), or other amplification reaction. Alternatively, the DNA sequence can be a hybridization probe. Preferably, the DNA sequence is at least 10 bases long; more preferably, the sequence is at least 50 bases long.

F. Insertion of the Cloned Gene for *S. pyogenes* DNase B into *E. coli* Expression Plasmid Δ33 Producing DNase B Under Regulation of the Bacteriophage λpL Promoter

The cloned gene for *S. pyogenes* DNase B can be transferred to the *E. coli* expression plasmid Δ33, which expresses the cloned gene under the control of the bacteriophage λ promoter pL. The *S. pyogenes* DNase B gene is preferably inserted into the expression plasmid by using PCR to attach modified ends to the DNase B gene from the λ2-6 clone. The following nucleotides can be used as primers for the PCR reaction following standard PCR procedures with *Thermus aquaticus* DNA polymerase:

A: 5' -T-A-A-C-G-G-A-T-C-C-G-A-A-T-C-T-A-C-T-T-G-G-A-T-C-A-A-G-A-C-G-G-G-T-T-T-T-T-C-T-3' (SEQ ID NO: 2)

B: 3' -T-C-T-T-T-T-T-C-G-T-T-A-C-T-A-A-C-G-G-C-A-G-T-A-A-C-G-G-G-G-C-C-C-A-G-C-T-G-G-G-C-C-5'. (SEQ ID NO: 3)

These primers can be used with the λ gt 11 DNase B clone 2-6 DNA as a template for amplification. The resulting amplification products can be digested with the endonuclease Bam HI and Sal I prior to insertion into the Δ 33 expression vector. This creates a translational fusion regulated by the pL promoter. A suitable strain of E. coli (C600C1⁺, gal K[']) is transformed with the inserted DNA, and bacteria containing the plasma can be selected by selection with ampicillin. DNA can be prepared from these colonies by standard minipreparation techniques, e.g., those described in F.M. Ausubel et al., "Current Protocols in Molecular Biology" (John Wiley & Sons, New York, (1987) § 1.6, followed by cutting the isolated plasmid with the appropriate restriction endonucleases (Bam HI and Sal I) to determine if the plasmid comprise the desired recombinant fragment. Plasmids of the desired construction can be introduced into an E. coli host strain that is subject to induction by the nalidixic acid protocol, as described in J. E. Mott et al., "Maximizing Gene Expression from Plasmid Vectors Containing the λ pL Promoter: Strategies for Overproducing Transcription Termination Factor ρ ," Proc. Natl. Acad. Sci. USA 82:88-92 (1985), incorporated herein by reference. It is known in the art that nalidixic acid damages DNA and induces recA protein, a recovery protein for E. coli. The recA protein has protease activity, which leads to inactivation of λ CI⁺ repressor; this inactivation leads to over-expression by the pL promoter. Other methods of activating transcription from the pL promoter can also be used. When nalidixic acid induction is used, substantial quantities of DNase B are secreted outside the cell.

II. PROPERTIES OF RECOMBINANTLY PRODUCED ENZYME

The recombinantly produced enzyme from λ 2-6 phage contains a leader peptide fused to the amino terminus of the DNase. This leader peptide has the sequence M-N-L-L-G-S-R-R-V-F-S-K-K-C-R-L-V-K-F-S-M-V-A-L-V-S-A-T-M-A-V-T-T-V-T-L-E-N-T-A-L-A-R (SEQ ID NO: 1).

Immunoinhibition assays (Example 7) demonstrate that recombinant S. pyogenes DNase B is inhibited by anti-DNase enzyme in human serum in a manner identical to non-recombinant DNase B enzyme, based on the ability of the DNase to use a DNA-dye complex as substrate.

III. MUTANTS OF RECOMBINANTLY PRODUCED DNASE B ENZYME

Another aspect of the invention is mutants or variants of the S. pyogenes DNase B gene which have altered DNase B activity. These mutant DNase B enzymes may have higher or lower levels of nuclease activity. These mutants include mutants of the protein whose amino acid sequence is shown in Figure 4 in which at least one of the following mutations occurs:

(a) a deletion of one or more amino acids from the sequence of Figure 4;

(b) an insertion of one or more naturally-occurring L-amino acids into the sequence of Figure 4; and

(c) replacement of at least one of the amino acids of Figure 4 with an alternative naturally occurring L-amino acid.

Such mutant DNase B proteins encoded by mutant DNase B genes can be used for their nuclease activity or for their immunogenicity. Preferably, when used for their immunogenicity, these mutants contain single amino acid changes which remove all nuclease activity, but maintain all significant immune epitopes, so that they substantially retain the antigenic reactivity of natural S. pyogenes DNase B enzyme. Thus, high level expression in E. coli can be achieved without altering human antibody reactivity with the altered DNase B. Such mutants or variants can be prepared according to techniques well-known in the art, such as those described in Sambrook et al., supra, Ch. 15, entitled "Site-Directed Mutagenesis of Cloned DNA." Such techniques include linker-insertion mutagenesis, linker-scanning mutagenesis, oligonucleotide-mediated mutagenesis with the polymerase chain reaction (PCR) technique, and growth in highly mutagenic strains.

When used for their nuclease activity, the mutant proteins will typically be functional equivalents of the recombinant DNase enzyme of Figure 4, as that term is defined above, but are not necessarily limited thereto.

5

IV. USE OF LEADER PEPTIDE FOR S. PYOGENES DNASE B ENZYME

The leader peptide for DNase B, with an amino acid sequence of M-N-L-L-G-S-R-R-V-F-S-K-K-C-R-L-V-K-F-S-M-V-A-L-V-S-A-T-M-A-V-T-T-V-T-L-E-N-T-A-L-A-R (SEQ ID NO: 1), can be used for expression and production of recombinant proteins in bacteria. A suitable process for the use of the leader peptide comprises:

(1) fusing the DNA coding for the protein to DNA coding for a leader peptide with an amino acid sequence of M-N-L-L-G-S-R-R-V-F-S-K-K-C-R-L-V-K-F-S-M-V-A-L-V-S-A-T-M-A-V-T-T-V-T-L-E-N-T-A-L-A-R (SEQ ID NO: 1) so that the fused DNA forms a recombinant protein with a single reading frame with the leader peptide being at the amino-terminus of the protein;

(2) introducing the fused DNA into the prokaryote; and

(3) expressing the fused DNA in the prokaryote so that the recombinant protein is produced in a recoverable quantity.

The bacterium can be Escherichia coli or, alternatively, a gram-positive bacterium such as Staphylococcus, Streptococcus, and Streptomyces.

Preferably, the recombinant protein is excreted by the prokaryote into its culture medium so that it can be recovered from the culture medium.

Methods for fusing the DNA segment coding for leader peptide to the gene for the protein to be produced are well-known in the art and include blunt-end ligation. Blunt-end ligation is typically performed with T4 ligase (V. Sgaramella & H.G. Khorana, "Studies on Polynucleotides. CXII. Total Synthesis of the Structural Gene for an Alanine Transfer RNA from Yeast.

Enzymic Joining of the Chemically Synthesized Polydeoxynucleotides to Form the DNA Duplex Representing Nucleotide Sequence 1 to 20," J. Mol. Biol. 72:427 (1972); V. Sgaramella & S.D. Ehrlich, "Use of the T4 Polynucleotide Ligase in the Joining of Flush-Ended DNA Segments Generated by Restriction Endonucleases," Eur. J. Biochem. 86:531 (1978)), and is preferably performed in the presence of condensing agents such as polyethylene glycol or hexammincobalt chloride.

Alternatively, if a suitable restriction endonuclease exists that generates cohesive ends and can cut both the portion of the DNA coding for the linker that corresponds to the carboxyl-terminus of the linker and the portion of the gene coding for the protein that corresponds to the amino-terminus of the protein, the restriction endonuclease can be used to generate cohesive ends for ligation.

V. PURIFICATION OF S. PYOGENES DNASE B ENZYME

A. Purification of Natural S. pyogenes DNase B

Another aspect of the present invention is an improved procedure for purification of natural S. pyogenes DNase B enzyme. This procedure was developed by using polyacrylamide gel analysis of the DNase B found in the commercial assay reagent and a comparison to the behavior on gel electrophoresis of the natural enzyme. The purification procedure employs the following steps, starting with a crude extract or other source of the enzyme: (1) absorption to and elution from diethylaminoethyl cellulose to produce a first eluate; (2) chromatography of the first eluate on phenyl agarose to produce a second eluate; (3) chromatography of the second eluate on heparin agarose to produce a third eluate; and (4) chromatofocusing of the third eluate to produce substantially purified DNase B enzyme. The chromatofocusing is preferably carried out on a mono-P column. Preferably, the purified DNase is further fractionated to remove ampholytes used during chromatofocusing using reverse-phase high-pressure liquid

chromatography on C4 with a gradient of 0.1% trifluoroacetic acid in water and 0.08% trifluoroacetic acid in acetonitrile.

5 The purification procedure results in substantially purified Streptococcus pyogenes DNase B enzyme substantially free of proteins other than Streptococcus DNase B enzyme and Streptococcus DNase B enzyme fused at its amino terminus with a leader peptide. The substantially purified protein is substantially free of mitogenic activity (See Example 6 below).

10 Purification results in two substantially purified DNase B fractions, differing in charge. Each of the fractions is substantially free of the other fraction and other proteins. These fractions are designated as Fraction I, which elutes from the chromatofocusing column at pH 8.55-8.4, and Fraction II, which elutes from the chromatofocusing column at pH 8.22-8.13. Molecular weight data obtained from mass spectroscopy (Example 3), indicates that the difference in molecular weights between Fractions I and II of the purified natural DNase B is consistent with a minor modification of an otherwise identical amino acid sequence. A possible modification is deamination, which would cause the appropriate pI shift.

20 The purified protein can be sequenced. The first 23 amino acids of both fraction I and II produced the following readable sequence: Q-T-Q-V-S-N-D-V-V-L-N-D-G-A-S-X-Y-L-N-E-A-L-A (SEQ ID NO: 4), where X represents tryptophan or lysine.

30 As detailed below, this sequence represents a means for designing probes suitable for hybridizing with at least a DNA sequence coding for the amino-terminal amino acid sequence of the gene.

35 B. Purification of Recombinantly Produced *S. pyogenes* DNase B Enzyme

Recombinant *S. pyogenes* DNase B, which is present at

a high level in the chimeric cells, can be purified by similar techniques. For example, the recombinant DNase B can be purified from phage lysate collected from E. coli infected with λ DNase B 2-6 phage by chromatography on Q-sepharose (trimethylaminomethyl agarose), ammonium sulfate precipitation, chromatography on heparin sepharose, and chromatography on Q-sepharose. The recombinant DNase B produced in E. coli transfected with recombinant plasmid Δ 33 expressing S. pyogenes DNase B from the pL promoter can be purified by chromatography on heparin sepharose, chromatography on Q-sepharose, and reverse phase high pressure liquid chromatography. Other purification methods are known and can be used by one skilled in the art.

VI. PREPARATION OF DNA PROBES CAPABLE OF HYBRIDIZING TO CLONED DNA

Another aspect of the invention is preparation of a single-stranded nucleic acid probe hybridizing with the DNA sequence coding for the amino-terminal 23 amino acids of the S. pyogenes DNase B enzyme with no greater than about a 30% mismatch. The nucleic acid probe can be RNA or DNA. Preferably, when the probe is DNA, the degree of mismatching is no greater than about 10% under standard stringent conditions, i.e., those described in F. Ausubel et al., in Current Protocols in Molecular Biology (Wiley-Interscience, New York, 1990).

Suitable sequences of such probes can be derived by using the codon usage table for enteric bacterial genes given for the relevant amino acids in Table 1.

TABLE 1
CODON USAGE FOR AMINO ACIDS IN AMINO-TERMINAL
REGION OF S. PYOGENES DNASE

<u>Gln (Q)</u>		<u>Thr (T)</u>	
<u>Codon</u>	<u>Frequency</u>	<u>Codon</u>	<u>Frequency</u>
CAG	0.86	ACC	0.55
CAA	0.14	ACU	0.35
		ACG	0.07
		ACA	0.04
 <u>Val (V)</u>		 <u>Ser (S)</u>	
<u>Codon</u>	<u>Frequency</u>	<u>Codon</u>	<u>Frequency</u>
GUU	0.51	UCC	0.37
GUA	0.26	UCU	0.34
GUG	0.16	AGC	0.20
GUC	0.07	UCG	0.04
		AGU	0.03
		UCA	0.02
 <u>Asn (N)</u>		 <u>Asp (D)</u>	
<u>Codon</u>	<u>Frequency</u>	<u>Codon</u>	<u>Frequency</u>
ACC	0.94	GAC	0.67
AAU	0.06	GAU	0.33
 <u>Leu (L)</u>		 <u>Gly (G)</u>	
<u>Codon</u>	<u>Frequency</u>	<u>Codon</u>	<u>Frequency</u>
CUG	0.83	GGU	0.59
CUC	0.07	GGC	0.38
CUU	0.04	GGG	0.02
UUG	0.03	GGA	0.00
UUA	0.02		
CUA	0.00		
 <u>Ala (A)</u>		 <u>Trp (W)</u>	
<u>Codon</u>	<u>Frequency</u>	<u>Codon</u>	<u>Frequency</u>
GCU	0.35	UGG	1.00
GCA	0.28		
GCG	0.26		
GCC	0.10		

<u>Tyr (Y)</u>		<u>Glu (E)</u>	
<u>Codon</u>	<u>Frequency</u>	<u>Codon</u>	<u>Frequency</u>
UAC	0.75	GAA	0.78
UAU	0.25	GAG	0.22

One example of such a probe is shown below:

Probe 1: C-A-P-U-A-C-N-C-A-R-T-N-W-S-N-A-A-Y-G-A-Y-G-T
(SEQ ID NO: 5).

5

In this sequence, R represents a purine (i.e., A or G), Y represents a pyrimidine (T or C), S represents G or C, W represents A or T, and N represents any of the four common deoxyribonucleotides (i.e., A, G, C, or T).

10

This probe, and other probes, can be synthesized by procedures well-known in the art, such as solid-phase DNA synthesis by the phosphotriester or phosphite triester methods, as disclosed, e.g., in "Nucleic Acids in Chemistry and Biology" (G. M. Blackburn & M. J. Gait, eds., IRL Press, Oxford, 1990), ch. 3. pp. 106-123.

15

VII. USE OF UPSTREAM PROMOTER ASSOCIATED WITH S. PYOGENES DNASE B

20

Another aspect of the present invention is the isolation and use of an upstream promoter originally associated with the S. pyogenes DNase B gene to express a protein other than DNase B. The detection of this promoter sequence is described below in Example 11.

25

The promoter sequence is retained in the λ 2-6 clone. This sequence includes a start site for transcription and sites substantially homologous to the consensus -10 and -35 sites for bacterial promoters (Example 11). This substantially purified promoter sequence is within the scope of the invention.

30

A method of using this promoter sequence for expressing a protein other than DNase B comprises:

35

- (1) separating the promoter originally associated with the S. pyogenes DNase B gene from the S. pyogenes DNase B gene;
- (2) operatively linking the promoter with a structural

gene for a S. pyogenes protein other than the gene for DNase B;
and

(3) expressing the protein encoded by the structural
gene.

5

The protein can be expressed in S. pyogenes or in a
prokaryote other than S. pyogenes, such as E. coli. The promoter
can be incorporated in a vector or a plasmid for expression of
a gene operatively linked to the promoter in the vector or
plasmid.

10

VIII. USE OF SUBSTANTIALLY PURIFIED DNASE B ENZYME

The present invention also encompasses several uses of
the substantially purified S. pyogenes DNase B enzyme, whether
purified from natural sources or produced by recombinant DNA
techniques.

15

A. Use of Enzyme for Preparation of Antibodies

20

Among the uses of the enzyme prepared by methods
according to the present invention is the preparation of
antibodies. The antibodies can either be polyclonal or
monoclonal. Preparation of both polyclonal and monoclonal
antibodies is described in E. Harlow and D. Lane, "Antibodies:
A Laboratory Manual" (Cold Spring Harbor Laboratory, Cold Spring
Harbor, New York, 1988). pp. 53-318. The resulting antibodies
can be used for detection of the S. pyogenes enzyme, i.e., in
suspected cultures.

25

B. Use of Enzyme for Detection of Anti-DNase B Antibody

30

An important use for the substantially purified S.
pyogenes DNase B enzyme of the present invention is the detection
of anti- S. pyogenes DNase B antibodies, such as in serum. As
described above, the presence of such antibodies is indicative
of active S. pyogenes infection and a warning signal that serious
suppurative sequelae may occur.

35

One method of detecting the anti-DNase B antibody employs the fact that the antibody is capable of inhibiting the activity of the enzyme. Such a method can comprise the following steps:

5 (1) providing a test sample suspected of containing anti-S. pyogenes DNase B antibody;

(2) adding a quantity of the S. pyogenes DNase B enzyme according to the present invention to the test sample, the quantity being sufficient to produce a detectable level of enzymatic activity in the absence of inhibition of the enzymatic activity by anti-DNase B antibody in the test sample; and

10 (3) determining the level of activity of DNase B enzyme in the test sample by performing an enzyme assay to detect and/or determine the anti-S. pyogenes antibody in the test sample.

15 The enzyme assay can be performed by standard methods, such as the DNA-dye complex degradation assay of Wampole Laboratories (Cranbury, NJ). This assay is based on the ability on the DNase to use a DNA-dye complex as substrate. This complex exhibits a maximum absorption wavelength of 642 nm. However, as the DNA-dye complex is degraded by the DNase, there is a shift in the maximum absorption wavelength and a decrease in the absorption at 642 nanometers. Other enzymatic assays are available, such as viscosimetric assays, which measure the ability of the enzyme to depolymerize long DNA molecules, thus greatly reducing the viscosity of solutions containing DNA. Alternatively, assays can be performed by using radioactive DNA as a substrate and quantitating the release of radioactivity after incubation. Other methods for the assay of deoxyribonuclease are well known in the art.

20 An alternative assay for anti-DNase B enzyme antibody in serum is an ELISA assay. This assay comprises:

25 (1) binding the S. pyogenes DNase B enzyme of the present invention to a solid support;

30 (2) reacting a test sample suspected of containing anti-S. pyogenes DNase B antibody with the S. pyogenes DNase B

enzyme to bind the antibody to the enzyme and thus to the solid support; and

(3) detecting the antibody bound to the solid support to detect and/or determine the antibody in the test sample.

5

ELISA procedures are well known in the art and are described, e.g. in P. Tijssen, "Practice and Theory of Enzyme Immunoassays" (Elsevier, Amsterdam, 1985). The solid support used is typically plastic, such as polystyrene, but other solid supports, such as nitrocellulose, can also be used. The detection of the bound antibody is typically performed by adding a second antibody specific for the first antibody; the second antibody does not bind the S. pyogenes DNase B enzyme. Such an antibody can be, for example, enzyme-labeled anti-human immunoglobulin G. The enzyme label is typically alkaline phosphatase, λ -galactosidase, glucose oxidase, or horseradish peroxidase. Such enzymes give products that have optical absorption in the visible spectrum, and can be detected either visually or with a spectrophotometer.

10

15

20

Other techniques of detecting and/or determining the formation of antigen-antibody complexes can also be used to assay anti-DNase B antibody in serum. These techniques detect an aggregated antigen-antibody complex, here an enzyme-antibody complex, by a change in light absorption or scattering. In general, such an assay comprises:

25

(1) preparing a buffered solution of the DNase B of the present invention;

30

(2) reacting the buffered DNase B solution with a test sample suspected of containing anti-S. pyogenes DNase B antibody; and

(3) detecting a reaction between the DNase B and the anti-DNase B antibody by observing and/or measuring a change in light absorption and/or light scattering in the solution.

35

The DNase B can be attached to higher molecular weight carriers, such as latex polymers, plastic or gel beads, and the

like.

5 In a number of applications, it is particularly advantageous to attach the enzyme to latex particles. This can provide several advantages, including stabilization of the enzyme and increased sensitivity of detection, either by turbidimetry or nephelometry. The latex agglutination assay format is popular for clinical immunoassays.

10 Many different coating methods can be used in attaching DNase B to latex particles. Some of the widely used methods are: (1) attachment by adsorption to latex particles; (2) attachment by covalent coupling via carboxyl groups on the latex particles; and (3) attachment by covalent coupling via aldehyde groups on
15 the latex particles.

The size of the latex particles used for attachment can range from about 15 nm to about 1000 nm in diameter, depending on the application.

20 The reaction between the DNase B and the anti-DNase B can be detected by nephelometry or turbidimetry. Another alternative method for detecting anti-DNase B antibody is capillary electrophoresis.

25 C. Other Uses

The recombinant protein can be used for vaccine development to immunize against S. pyogenes in susceptible individuals, and also can be used as an aerosol in the treatment
30 of lung viscosity symptoms in diseases such as cystic fibrosis when the viscosity is due to exudates containing high concentrations of DNA.

35 For use as a vaccine, a quantity of a purified S. pyogenes DNase B enzyme according to the present invention is administered to a mammal. The quantity is sufficient to stimulate production of antibodies specific for S. pyogenes DNase

B.

Administration is typically by injection, and can be intravenous, intramuscular, subcutaneous, intradermal, or by other routes. The DNase B enzyme can be administered with an adjuvant to increase the immune response. Suitable adjuvants are well known in the art and include complete and incomplete Freund's adjuvant, as well as aluminum hydroxide with or without heat-killed Bordetella pertussis bacteria. The enzyme can be aggregated, precipitated, or coupled to insoluble matrices.

One particularly suitable enzyme according to the present invention for use in immunization is a mutant enzyme in which the DNase activity is completely or substantially eliminated and the antigenicity is wholly or substantially retained.

A method of use of enzymatically active DNase B enzyme according to the present invention can comprise:

- (1) generating an aerosol of purified enzymatically active DNase B enzyme according to the present invention; and
- (2) administering the aerosol to a patient with cystic fibrosis in a quantity sufficient to reduce lung fluid viscosity in the patient.

Administration of the aerosol can be performed using inhaler devices well-known in the art and frequently used to deliver steroids and other drugs to the respiratory tract by inhalation.

EXAMPLES

The following examples are intended for illustrative purposes only and are not intended to limit the invention.

Example 1

Cloning of Streptococcus Pyogenes DNase B Gene

The S. pyogenes DNase B gene was identified by an activity based colorimetric detection of nuclease activity produced from a recombinant λ bacteriophage. The phage was a product of a λ library containing sheared DNA purified from S. pyogenes (Lancefield Group ATCC No. 14289) genomic DNA.

Preparation of Chromosomal DNA from S. pyogenes

S. pyogenes strain ATCC 14289 was streaked onto Todd Hewitt agar plates and incubated at 37°C for two days. A single colony was used to inoculate one liter of Todd Hewitt broth with 10% calf serum. The culture was allowed to grow to high density at 37°C with shaking for about 36 hours.

The cells were collected by centrifugation in a Beckman J6 centrifuge at 3500 rpm at 4°C for 45 minutes. The cell pellet was resuspended in 25 ml of 40 mM Tris, pH 7.5, 1 mM EDTA. The proteolytic enzyme achromopeptidase (60 mg) (Wampole), in 1 ml buffer, was added and the mixture was incubated at 65°C for one hour. No lysis was visible. A total of 20 ml of 10% SDS was then added, and incubation was continued for one hour. Lysis was very apparent. Fifty milliliters of buffer was then added to reduce the concentration of SDS to 2.5%.

The mixture was extracted twice with an equal volume of phenol, followed by one extraction with phenol/chloroform (1:1). The DNA in the aqueous phase was precipitated by ethanol. The DNA was recovered by centrifugation. The pellet was resuspended in 4 ml of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (TE). RNase A (50 μ l at 10 mg/ml) was added and the mixture was incubated at 37°C for three hours.

The DNA was further purified in a cesium chloride gradient. The final concentration of DNA was about 0.5 mg/ml.

Construction of S. pyogenes Library in λ gt11

5 The isolated chromosomal DNA (300 μ ml) was added to 200 μ l of TE buffer. The mixture was passed through a 1 ml syringe with a 25-gauge needle about 300 times to shear the DNA to an average size of 6 kb.

10 The sheared DNA (150 μ l) was treated with E. coli ligase to repair existing nicks in the DNA which might otherwise have become gaps with subsequent manipulations. To 150 μ l of DNA was added 20 μ l of 10 x E. coli ligase buffer (0.5 M Tris-HCl, pH 7.6, 100 mM MgCl₂, 100 mM dithiothreitol, and 500 μ g/ml bovine serum albumin), 20 μ l of NAD⁺ (36 mM) and 7 μ l of E. coli ligase (New England Biolabs, Beverly, Mass., 4 units/ μ l) was added to 15 the DNA and the mixture was left at room temperature for four to five hours. The ligase was heat killed at 65°C for 15 minutes. The DNA was precipitated by ethanol.

20 Eco RI sites in the ligase-treated DNA were methylated with Eco methylase following the protocol of the manufacturer (Promega, Madison, Wis.). This was done to block internal Eco RI sites whose cleavage would interfere with the cloning procedure.

25 The sheared ends of the DNA were repaired with T4 DNA polymerase by adding 30 μ l of 0.1 M MgCl₂, 20 μ l of 2.5 mM of each of the four deoxyribonucleoside triphosphates, and 2 μ l of T4 DNA polymerase (3000 U/ml) to the DNA mix after methylation. The reaction was carried out for 15 minutes at room temperature. 30 The mixture was extracted once with phenol/chloroform and then with ether. The DNA in the aqueous fraction was then precipitated with ethanol.

35 Eco RI linkers were ligated onto the DNA. The linkers used were octamers from New England Biolabs. After linker ligation, the DNA was digested with an excess of Eco RI restriction endonuclease enzyme. DNA of the desired size range,

namely 6-8 kb, was purified from an agarose gel after electrophoresis. The DNA was concentrated by ethanol precipitation and was then ready for ligation into λ gt11.

5 Approximately 2 μ g of sheared DNA was ligated with 1 μ g of λ gt11 arms previously digested with Eco RI restriction endonuclease and in which the terminal phosphate residue had been removed by treatment with alkaline phosphatase. The ligation was carried out with bacteriophage T4 ligase in a total volume of 5 μ l. The ligation reaction was performed at 4°C overnight.

10 The entire ligation mix was packaged in vitro using the Promega (Madison, Wis.) packaging extract. One μ l of packaged phage was plated on a lawn of Y 1090 E. coli in the presence of isopropylthio- β -D galactoside (IPTG) and the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactoside) (Xgal) approximately 5% of the plaques were blue. The packaging efficiency was approximately 10^6 plaques per μ g of DNA.

20 Screening for λ Recombinant Clones with Nuclease Activity

25 The non-amplified library (10 μ l) was plated with 0.1 ml of an overnight culture of LE 392. After five hours, the plates were overlaid with 0.5 x BBL DNase test agar plus 0.01% toluidine blue plus 10 mM MgCl₂. A total of 10 plates was screened. Forty-four pink plaques (potentially nuclease positive) were rescreened. Nine of the 44 pink plaques consistently rescreened as positive for nuclease activity.

30 Because the production of S. pyogenes DNase is deleterious to the host E. coli bacteria, the plaque size of these nuclease positive clones was much smaller than for nuclease negative clones. Accordingly, there was selection pressure for accumulating mutations that would lower the nuclease activity, which complicates the task of isolating a stable nuclease positive clone.

One of the advances of the selection and screening procedure of the present invention is to lower the selection pressure allowing stable nuclease positive clones. To do this, E. coli strain Y1090 without the plasmid pMC9 was used as the host for nuclease carrying phage. Plate lysates were used to generate stocks to plaque purify the clones. For this procedure, the host and phage were plated directly on 0.5 x BBL DNase test agar plus 0.01% toluidine blue plus 10 mM MgCl₂ directly instead of overlaying after five hours of incubation.

Lysates of the nine recombinant clones were analyzed on SDS-polyacrylamide gels containing DNA. The nuclease in all nine clones retained their activity after SDS denaturation and all have the same apparent molecular weight, approximately 25 kd.

These nine lysates were analyzed on the PhastGel system with IEF 3-9 gels for electrofocusing. After electrophoresis, the gels were overlaid with 3.5 ml DNase substrate (Streptonase B kit) (Difco, Detroit, Michigan) in 1% agarose in TAE (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 8). The activity bands for all 9 lysates at the edge of the basic end of the gel, suggesting a very high pI for the cloned nuclease. This also suggested that all nine clones contained the same gene.

In particular, one phage showing DNase activity, designated as 2-6 was analyzed further. The λ DNase 2-6 clone was analyzed with restriction endonuclease analysis to characterize the DNA fragment. The S. pyogenes genomic insert in the λ vector in the 2-6 clone was approximately 5.2 kb. The location of the nuclease gene was determined by subcloning smaller regions of the DNase 2-6 clone back into λ gt 11 and testing the subclones for nuclease activity. Figure 2 shows the location of the various subclones and their nuclease activity. Subclones 1 and 4 produced nuclease activity but were very unstable. Subclones 2 and 3 lacked nuclease activity but were stable. The results of this subcloning indicated that at least part of the DNase B gene resides in the internal Sac I/Eco RI fragment. The amino-terminal sequence from the DNase B protein

was used in conjunction with the genetic code to generate a set of degenerate oligonucleotides that was used to hybridize to the DNase 2-6 insert and some of the subclones. These oligonucleotides hybridized to the 3.5 kb Eco RI fragment in DNase B 2-6 and the Sac I/Eco RI fragment in subclone 3. This data, together with the subcloning data, suggest that the transcription of the nuclease gene is very likely from left to right as diagrammed, and the Sac I site is within the DNase B gene.

Mapping of the S. pyogenes DNA adjacent to the 5.2 kb insert was done by genomic DNA blot hybridization. The 3.5 kb and 1.5 kb Eco RI fragments of the λ DNase 2-6 DNA were gel purified and labeled with ^{32}P by random priming. The same genomic blots were hybridized with the two probes consecutively. A partial restriction endonuclease map of the insert and its neighboring region in the S. pyogenes chromosome is shown in Figure 1.

Example 2

Sequencing of the Clone 2-6 Containing S. pyogenes DNase B

Nucleotide sequence analysis was performed on clone 2-6 by the dideoxynucleotide chain termination method of Sanger et al., supra. Sequence analysis was initiated by priming synthesis from within the λ gt11 phage of clone 2-6 across the suspected region of DNase activity. The results of sequencing are shown in Figure 3. The S. pyogenes DNase B is within the first full open reading frame of the sequence.

Example 3Purification of Native *S. pyogenes* DNase B

5 Native *S. pyogenes* DNase B was purified using a commercial DNase B assay reagent as a marker of the correct nuclease. In other words, polyacrylamide gel electrophoresis results obtained with the DNase B in the commercial kit was compared to the results from gel electrophoresis in extracts
10 produced from *S. pyogenes* ATCC No. 14289. The purification procedure included: batch absorption on DE-23 diethylaminoethyl cellulose (Whatman) (2) chromatography on phenyl Sepharose® (Pharmacia, Uppsala, Sweden); (3) chromatography on heparin Sepharose® (Pharmacia); and (4) mono-P chromatofocusing
15 (Pharmacia).

Bacterial Cultures

Streptococcus pyogenes ATCC NO. 14289 (American Type
20 Culture Collection, Rockville, Maryland), derived from A. Bernheimer C203S (non-M containing variant of C203) was used as the bacterial source for the collection of DNase B-containing culture media, the enzyme being secreted into the culture media by the bacteria. Volumes of brain heart infusion media (1 liter)
25 (Difco Laboratories, Detroit, Michigan) supplemented with 0.01% washed goat red blood cells were inoculated with 1 ml of a fresh overnight culture. These cultures were grown for 20 hours with moderate agitation (300 rpm) at 37°C in 2 liter Erlenmeyer flasks. Prior to purification the culture medium was clarified
30 and sterilized by filtration using a Pellicon filter (0.22 μ m Durapore GVLP membrane) followed by filtration through an 0.45 μ m disposable filtration apparatus (Nalgene, Nalge Co., Rochester, New York). Approximately 105 liters of culture media was processed with this procedure.

35

Batch Absorption to Diethylaminoethyl Cellulose

5 The clarified media was concentrated by ultrafiltration using the Pellicon apparatus and a 10 K membrane (PLGC, regenerated cellulose) with a filter area of about 0.46 m² at a flow rate of 120 ml/min, and a pressure of 20 lbs per square inch (1.4 Kg/cm²). The initial volume of 105 liters of media was eventually concentrated to 4 liters with a protein concentration of 2.3 mg/ml.

10 Diethylaminoethyl cellulose (DEAE-cellulose) (DE23, Whatman, England) was regenerated by washing with 15 volumes of 0.5 M HCl followed by a second wash with 15 volumes of 0.5 M NaOH. After a repetition of the washing with sodium hydroxide, 15 the DEAE-cellulose was washed with water until neutral. Finally, the cellulose was equilibrated overnight in TMC buffer (1 mM Tris, 1 mM MgCl, 1 mM CaCl₂, pH 7.5).

20 The equilibrated wet cellulose (100 g) was added to 500 ml of concentrated S. pyogenes media supernatant. The mixture was shaken at 300 rpm for 20 minutes at 4°C prior to centrifugation at 3500 rpm for 45 minutes. The cellulose was washed with 450 ml of TMC buffer and the two supernatants were combined.

Chromatography on Phenyl Sepharose

25 The supernatants from diethylaminoethyl cellulose batch absorption were clarified by filtration through a 0.45 μm membrane. Ammonium sulfate was added to 0.8 M prior to passage 30 through phenyl Sepharose CL 45 (Pharmacia, Uppsala, Sweden) equilibrated with 0.8 M ammonium sulfate, 20 mM sodium phosphate (pH 8.0).

35 The 80-ml phenyl sepharose column was loaded at 1.85 ml/min with 1100 ml of sample at a concentration of 258 μg/ml. The DNase activity was collected in the flow-through prior to

concentration by ultrafiltration using a 10-kd membrane (Diaflo YM10, Amicon Division, W. R. Grace & Co.) The final protein concentration was 0.245 mg/ml, in 55 ml.

5 Chromatography on Heparin Sepharose

The concentrated effluent from the phenyl-sepharose column was dialyzed against Heparin Buffer A (20 mM HEPES, pH 7.9, 2 mM dithiothreitol, 10 mM MgCl₂, 0.2 mM EDTA, 0.1 NaCl, 10% glycerol). A heparin Sepharose CL-6B (Pharmacia) column (80 ml) was equilibrated with the Heparin Buffer A prior to loading at a flow rate of 1.0 ml/min. After washing the column with three volumes of heparin buffer A, a gradient between 0% and 100% buffer B was run at a flow rate of 2.2 ml/min. Buffer B was the same as buffer A except that the concentration of sodium chloride was 1.0 mole/l. The DNase activity eluted at 350 mM NaCl in volume of approximately 250 ml. The DNase activity was concentrated by ultrafiltration.

20 Mono-P Chromatofocusing

The concentrated DNase fraction was dialyzed against 25 mM diethanolamine, pH 9.5 prior to chromatofocusing. The mono P 5/20 column (Pharmacia, Piscataway, N.J.) equilibrated in the loading buffer (25 mM ethanolamine, pH 9.5), was injected with 500 μ l of sample and washed with 9 ml of loading buffer. The column was eluted with 100% buffer B (10% polybuffer 96 (Pharmacia), pH 6.0). The total volume eluted was 34 ml; fractions of 0.5 ml were collected. Two peaks of activity were collected at pH 8.55-8.4 (fractions 25-29), designated herein as Fraction I, and 8.22-8.13 (fractions 34-35), designated herein as Fraction II. The collected fractions were analyzed by isoelectric focusing activity gels, silver staining, and by SDS-polyacrylamide gel electrophoresis.

35

Reverse Phase High-Pressure Liquid Chromatography

Peak fractions from the chromatofocusing column were further purified to remove the ampholytes used for chromatofocusing by reverse phase high pressure liquid chromatography using a C4 column (Beckman System Gold Instrument, Beckman Instruments, Fullerton, California). Samples were loaded in buffer A (0.1% trifluoroacetic acid in water) and a gradient of 0%-100% buffer B (0.8% trifluoroacetic acid in acetonitrile) was used to elute the column at a flow rate of 1 ml/min. Those proteins eluted in 65% buffer B in a volume of about 1 ml.

SDS and Isoelectric Analysis

SDS-polyacrylamide gel analysis of all samples was performed using the PHAST System (Pharmacia LKB, Piscataway, New Jersey) automated instrument. SDS-polyacrylamide gel electrophoresis was performed on the PhastGel 10-15% gels. Isoelectric gels were run using the PhastGel IEF 3-9 gels. Silver staining of both the SDS and the isoelectric gels was performed using the PhastSystem automated staining device (Pharmacia LKB). Activity assays of the DNase samples on the isoelectric focusing gels were performed by overlaying the gels after electrophoresis with 5 ml of a 1% melted agarose solution containing phosphate buffered salts and 1 ml of reconstituted DNase substrate dye (Wampole). Incubation of the IEF gels with the substrate overlay at room temperature resulted in the detection of activity by the conversion of the blue substrate dye to a pink color centered around the nuclease activity. Activity assays of SDS-denatured samples were performed using an SDS-14% polyacrylamide gel that was polymerized in the presence of 500 $\mu\text{g/ml}$ herring testes DNA. After electrophoresis, the gels were rinsed with water and equilibrated with 40 mM Tris-HCl, pH 7.5, 2 mM MgCl_2 , 0.02% sodium azide for 2 hours at 37°C. Ethidium bromide was added to 1 $\mu\text{g/ml}$ in order to observe the nuclease activity visible as a result of the degradation of the DNA by the nuclease.

Protein Sequencing

The amino-terminal sequences of Fractions I and II of the purified DNase were determined using an Applied Biosystems (Foster City, California) 477 sequenator. Samples of each of the purified enzymes (Fractions I and II) were loaded on to an Applied Biosystems (Foster City, CA) 470 Protein Sequencer. The first 23 amino acids of both Fraction I and II produced the following readable sequence: Q-T-Q-V-S-N-D-V-V-L-N-D-G-A-S-X-Y-L-N-E-A-L-A (SEQ ID NO: 4), where X stands for an amino acid that cannot be definitely identified, but is most likely either tryptophan or lysine.

Mass Spectroscopy Analysis

Ion-spray mass spectral analysis was performed on recombinant DNase B (Example 1) and on Fractions I and II of the purified native DNase B using the Finnigan MAT TSQ 700 triple-stage quadrupole mass spectrometer equipped with the Finnigan Electrospray ionization system. Samples were prepared by reverse phase fractionation using a C4 column as described above. The DNase B proteins eluted at 65% Buffer B and were lyophilized for storage. Prior to injection at a flow rate of 1 μ l/min, the samples were solubilized in acetonitrile-water-acetic acid (50:50:1).

The molecular weights determined by mass spectroscopic analysis are as follows: recombinant DNase B (Example 1) -- 25,549; Fraction I of purified natural DNase B -- 25,390; Fraction II of purified natural DNase B -- 25,397. These results are consistent with the nucleotide and amino acid sequencing results, which indicate that the recombinant DNase B has one additional amino acid at the amino terminus. The difference in molecular weights between Fractions I and II of the purified natural DNase B is consistent with a minor modification of an otherwise identical amino acid sequence. A possible modification is deamination, which would cause the appropriate pI shift.

Example 4Purification and Amino-Terminal Sequence Analysis of
Recombinant *S. pyogenes* DNase B Produced From
Bacteriophage λ 2-6 Clone

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The recombinant DNase B protein in the λ DNase B 2-6 phage lysate was identified on an SDS-polyacrylamide gel by Western blot analysis. Rabbit antibody against commercial DNase B was used to detect the presence of recombinant DNase B. Only one protein band was detectable. Coomassie blue staining of an SDS-polyacrylamide gel suggests that the recombinant DNase B protein was about 5% of the total protein in the lysate. Only one nuclease was detected in a SDS-DNA-polyacrylamide gel system. The nuclease has a apparent molecular weight of 25,000 daltons.

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The purification of the recombinant DNase B protein was monitored using SDS-polyacrylamide gel and a nuclease activity assay using the substrate used for a control in the commercial DNase B assay kit. The purification procedure included: (1) chromatography on Q-sepharose (trimethylaminomethyl agarose); (2) ammonium sulfate precipitation; (3) chromatography on heparin-agarose; and (4) chromatography on Q-sepharose. Two liters of a λ DNase B 2.6 phage lysate was prepared as an overnight culture on Luria broth supplemented with 10 mM MgCl₂. The supernatant was collected after centrifugation of the culture in a Beckman Instruments (Fullerton, CA) centrifuge at 3635 x g at 4°C for 45 minutes to remove cell debris (the volume of supernatant was 1900 ml).

35

The lysate was filtered through a 0.45 μ m filtration unit to remove residual bacteria and cell debris. This filtrate was then passaged through an approximately 200-ml column of Q-sepharose (Pharmacia, Piscataway, NJ, which had been equilibrated with 20 mM Tris-HCl, pH 7.5, 1 mM EDTA. The flow-through from the column was collected. To this fraction, ammonium sulfate

was added slowly to a final concentration of 80% at room temperature to concentrate the lysate. The desalted proteins were centrifuged at 15,000 x g for 30 minutes.

5 Glycerol was added to the dialyzed proteins to a final concentration of 10%. This preparation was filtered through a 0.45 μm filtration unit. Conductivity of the protein preparation was determined, and the protein preparation was diluted with 20 mM Tris-HCl, pH 7.5, so that the conductivity was the same as
10 that of a solution of 20 mM Tris-pH 7.5, 25 mM NaCl, 10% glycerol (Buffer A). The final volume was 1800 ml.

 This sample was loaded on to a heparin-sepharose column (approximately 100 ml) on a Pharmacia FPLC system at a flow rate
15 of 120 ml/hr. The column was washed with 400 ml of Buffer A. The DNase B was eluted with one liter of a gradient from 25 mM to 500 mM NaCl in Buffer A. The DNase activity eluted at approximately 125 mM NaCl in a volume of approximately 175 ml.

20 The DNase fraction eluted from the heparin agarose column was dialyzed against 20 mM Tris-HCl, pH 8.5, and was loaded on to an approximately 175-milliliter Q-sepharose column that had been equilibrated in 20 mM Tris-HCl, pH 8.5. The flow-
25 through from the Q-sepharose column was collected and analyzed by isoelectric focusing activity gels, silver staining, and by SDS-polyacrylamide gel electrophoresis. The preparation of recombinant DNase B protein was 99% homogeneous. The protein concentration in the final eluate (110 ml) was about 100 $\mu\text{g}/\text{ml}$.
30 This is equivalent to a yield of about 5.5 mg/liter of culture. The final product was then subjected to reverse phase high-pressure liquid chromatography, as described above in Example 3.

35 The amino-terminal sequence of purified recombinant DNase B was determined using a Beckman Microsequencing System 2020/Gold. The amino acid sequence was identical to that of

natural S. pyogenes DNase B, except that the amino-terminus was arginine (R), and was R-Q-T-Q-V-S-N-D-V-V-L-N-D-G-A-S-K-Y-L-N-E-A-L-A-W-T-F-N-D-S-P-N-Y-Y-K-T-L-G (SEQ ID NO: 6). This arginine arose from the process of producing the recombinant DNase B.

5

Mass spectroscopic analysis of the DNase B showed that the DNase was homogeneous, with an apparent molecular weight of 25,549.

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Example 5

Cloning and Expression of S. pyogenes DNase B Enzyme in an Escherichia coli Plasmid Under Regulation of the pL Promoter

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An additional genetic construction was made to demonstrate the regulated expression of the S. pyogenes DNase B gene using a plasmid vector incorporating the bacteriophage λ promoter pL. This construction was made by using the polymerase chain reaction (PCR) to incorporate modified ends to the DNase B gene in the λ 2-6 clone. The following oligonucleotides were designed and synthesized on the Pharmacia Gene Assembler Plus DNA synthesizer following the manufacturer's recommendations:

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A: 5'-T-A-A-C-G-G-A-T-C-C-G-A-A-T-C-T-A-C-T-T-G-G-A-T-C-A-A-G-A-C-G-G-G-T-T-T-T-T-C-T-3' (SEQ ID NO: 2)

30

B: 3'-T-C-T-T-T-T-T-C-G-T-T-A-C-T-A-A-C-G-G-C-A-G-T-A-A-C-G-G-G-G-C-C-C-A-G-C-T-G-G-G-C-C-5' (SEQ ID NO: 3).

35

These oligonucleotides were used as primers in a PCR reaction using the AmpliTaq kit (Perkin-Elmer-Cetus, Norwalk, CT), according to the manufacturer's instructions. The final concentration of $MgCl_2$ was adjusted to 4 mM, and a 20 cycle reaction was performed (37°C, 2 minutes; 72°C, 3 minutes; 95°C, 2 minutes) using the Perkin-Elmer 480 thermal cycler. DNA of the λ gt 11 clone 2-6 (100 ng) was used as a template along with 200

5 μ M of each primer. The resulting amplified product was further digested with Bam HI and Sal I prior to insertion into the Δ 33 expression vector. These manipulations created a translational fusion with the sequence as shown in Figure 5, which is regulated by the λ pL promoter.

10 C 600 Cl⁺, galK bacteria were transformed with the ligation mixture and plated on to LB-Amp plates. Thereafter, a minipreparation of DNA was made (F.M. Ausubel et al., eds., "Current Protocols in Molecular Biology" (John Wiley, 1987), Section 1.6), followed by cutting the plasmid with the enzymes Bam HI and Sal I to determine if the plasmid comprised the recombinant DNase B fragment. Plasmids of the desired construction were further transformed into the AR120 host strain. 15 These host cells with plasmids comprising the recombinant DNase B were then subjected to induction via the nalidixic acid protocol (Mott et al., supra). Colonies comprising the transformed AR120 were lifted from the agar plates and inoculated in Superbroth (base: 12g tryptone, 24 g yeast extract, 5 ml 20 glycerol, 900 ml distilled H₂O; salts per liter of base: 1.7 g KH₂PO₄, 15.8 g K₂HPO₄ (anhydrous), 100 ml distilled H₂O), plus 100 μ g/ml ampicillin and grown at 37°C until the optical density of the culture at 650 nm equalled 0.4.

25 Thereafter, nalidixic acid was added to the inoculated mixture at a final concentration of 60 μ g/ml. The culture was incubated at 37°C for about 8 hours or, alternatively, overnight (approximately 16 hours). All cell fractions were assayed for DNase B activity including supernatant from the culture, 30 sonicated cell pellets, and supernatants from the sonicated cell pellets.

35 For the overnight induction, DNase B was secreted outside the E. coli cells. The 8-hour induction had most of the DNase B secreted outside the cell, with approximately 30% inside, recovered in the sonicated supernatant. The quantities of DNase B were great enough to be visualized by Coomassie brilliant

blue stain on polyacrylamide gel electrophoresis.

Example 6

5 Purification of Recombinant S. pyogenes
 DNase B Produced in E. coli Under Regulation
 of the pL Promoter

10 A quantity (6 liters) of a recombinant DNase B clone
was grown in superbrotth and induced overnight as described in
Example 5. The supernatant was harvested and concentrated with
a Pellicon concentrator using a 10K membrane; concentration
yielded a volume of 600 ml.

15 The concentrated extract was dialyzed against heparin
buffer A (20 mM HEPES, pH 7.9, 2 mM dithiothreitol, 10 mM Mg Cl₂,
0.2 mM EDTA, 0.1 M NaCl, 10% glycerol). The heparin column was
loaded, run, and eluted as in Example 3.

20 The eluate from the heparin column was dialyzed in
20 mM ethanolamine, pH 8.5. Small quantities of extraneous
proteins were absorbed from the DNase B preparation by batch
absorption onto Q-sepharose. A quantity of Q-sepharose (100 ml)
was equilibrated with 20 mM ethanolamine, pH 8.5, and added to
25 100 ml of the heparin DNase B fraction. The Q-sepharose was
allowed to bind to the extract in a batch procedure for 20
minutes at 4°C. After binding, the Q-sepharose was filtered
through a 0.45 µm filtration unit. The resin was finally washed
with 50 ml of 20 mM ethanolamine, pH 8.5 for 20 minutes, prior
30 to separation by centrifugation. The two eluates from this
procedure were combined and analyzed by reverse phase
chromatography, amino acid sequencing, and mass spectroscopic
analysis. For reverse phase chromatography, 1 ml of the purified
DNase B was passed through a C4 column and eluted at 65% Buffer B
35 in a volume of 1 ml. The same buffers were used as for the
purification of the native DNase B in Example 3.

The amino acid sequence was determined using a Beckman Microsequencing System 2020/Gold. The amino acid sequence was R-Q-T-Q-V-S-N-D-V-V-L-N-D-G-A-S-K-Y-L-N-E-A-L-A-W-T-F-N-D-S-P-N-Y-Y-K-T-L-G (SEQ ID NO: 6).

5

Example 7

Preparation of DNA Probe Corresponding to Amino-Terminal Sequence of DNase B Enzyme

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Using the codon usage for highly expressed genes in enteric bacteria on the VAX GCG program (Table 1), the following degenerate probe was prepared: C-A-P-U-A-C-N-C-A-R-T-N-W-S-N-A-A-Y-G-A-Y-G-T (SEQ ID NO: 5). In this sequence, R is a purine (i.e. A or G), Y is a pyrimidine (T or C), S is G or C, W is A or T, and N is any of the four common deoxyribonucleotides. This probe hybridized efficiently to λ gt11 clone 2.6, confirming that the native DNase B protein was derived from the cloned gene.

20

Example 8

Inhibition of Recombinant DNase B by Anti-DNase B Antibody

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To show that the recombinant *S. pyogenes* DNase B is equivalent in its properties to natural DNase B, an immunoinhibition assay was performed. The recombinant DNase B was compared with commercially available natural DNase B in an inhibition assay using control positive human serum containing anti-DNase B antibody. The assay used was based on the ability of the DNase B to use a DNA-dye complex as substrate. This complex exhibits a maximum optical absorption at 642 nm. However, as the DNA-dye complex is degraded by the DNase, there is a shift in the maximum wavelength of absorption, and enzyme activity is indicated by a decrease in the measured absorption at 642 nm. As shown in Figure 6, the recombinant enzyme is

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inactivated in an identical manner to the natural S. pyogenes DNase B by human serum containing anti-DNase B enzyme as the result of an immune reaction against naturally occurring S. pyogenes DNase B.

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Example 9

Determination That Transcription of the DNase B Gene Is Occurring From a Streptococcus Promoter in the A2-6 Clone

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As shown in Example 4, there was a high level of expression of the DNase B gene from the λ 2-6 clone. In order to determine the start site of the strong bacterial promoter responsible for this expression, an in vitro runoff transcription assay was performed using E. coli RNA polymerase. This assay allows one to determine a precise base of transcriptional initiation by comparing the length of a transcriptional RNA runoff with a Sanger dideoxy sequencing ladder. This assay provides strong evidence for the start site of transcription in E. coli. Comparison with the known transcriptional start sites of a variety of Streptococcus further verifies this site to be the region responsible for streptococcal transcription (J. Ferretti & R. Curtiss, "Streptococcal Genetics" (1987), p. 293 ("Compilation of Nucleotide Sequences that Signal the Initiation of Transcription and Translation in Streptococci")).

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In a runoff transcription reaction, the RNA polymerase recognizes promoter regions and initiates transcription. The enzyme eventually falls off the end of the template, hence this is runoff transcription. This is a standard method for studying transcription start sites.

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A PCR fragment which includes the upstream region of the DNase B gene was made as a template for an in vitro runoff transcription reaction with E. coli RNA polymerase. Using two oligonucleotides, oligonucleotide #246 at positions 298 to 280 and oligonucleotide #267 (not shown in Figure 3), a PCR DNA

product of approximately 290 base pairs was made and the fragment was purified after gel electrophoresis. The runoff transcription reaction was performed in 30 mM Tris pH 8, 120 mM KCl, 4 mM MgCl₂, 10 mM 2-mercaptoethanol, 4 mM spermidine, 0.4 mM ATP, 0.4 mM CTP, 0.4 mM GTP, 0.08 mM UTP, 80 units RNAsin (Promega), 1 unit RNA polymerase (Promega) and 5 μl [³²P] UTP in a total volume of 100 μl. The mixture was incubated at 37°C for 30 minutes. In order to stop the reaction, 10 μl of 0.5 M EDTA was added.

The sample was diluted and electrophoresed on a sequencing gel. In order to accurately determine the size of the transcript, a sequencing reaction using oligonucleotide 246 on 2-6 DNA was performed. The reaction was done using the GIBCO/BRL (Bethesda, MD) cycle sequencing kit. The starting point of the sequencing ladder is the same as the runoff point of the runoff transcript. By analyzing the transcription product along with the sequencing ladder in a urea polyacrylamide gel, the location of the transcription initiation site was determined.

Figure 7 shows the DNA sequence upstream of the open reading frame and the consensus sequence of an *E. coli* promoter (D.K. Hawley & W. R. McClure, Nucl. Acids Res. 11:2237-2255 (1983)). The transcription data suggests that there are two possible start sites, position 96 and 97, for RNA polymerase. These sites are marked by an asterisk in Figure 7. The -35 and -10 regions are underlined.

Example 10

Equivalence of Purified Recombinant S. pyogenes DNase B with Natural DNase B in Reaction with Anti-DNase Antibody in Human Serum Samples

To show that recombinant DNase B was substantially equivalent with natural DNase B in the form of commercial

Streptonase B, in their reaction with anti-DNase antibody in human serum samples, the purified DNase B enzyme was used in place of the commercial Streptonase B in the Streptonase B assay. Ten patient samples from Boston Biomedica (Boston, MA) were tested following the directions provided in the Streptonase B diagnostic kit. The same samples were also tested using purified recombinant DNase B diluted to give similar nuclease activity as the reconstituted Streptonase B. The results are shown in Table 2 and graphed in the form of a correlation curve in Figure 8.

TABLE 2

EQUIVALENCE OF RECOMBINANT DNase B WITH ISOLATED DNase B
IN DETERMINATION OF ANTI-DNase B ANTIBODY TITER

As can be seen, the correlation between the results using commercial Streptonase B and the purified recombinant DNase is quite high. Thus, purified recombinant DNase B reacts in substantially the same manner with anti-DNase antibody found in serum as does commercial Streptonase B.

Example 11

Lack of Mitogenic Activity of Purified Natural DNase B

In order to determine whether the purified natural DNase B had mitogenic activity in a human lymphocyte mitogenic assay, various fractions of the purified natural S. pyogenes DNase B were tested in a mitogenic assay similar to the procedure used by T. Yutsudo et al., "A New Type of Mitogenic Factor Produced by Streptococcus pyogenes," FEBS Lett. 308: 30-34 (1992). For the testing of DNase B for mitogenic activity, human lymphocytes were isolated using a Ficoll-Paque (Pharmacia) one-step gradient procedure performed as described by the manufacturer. Lymphocytes were plated in a microtiter plate (96 Wells) at a concentration of 10^5 cells/well. After three days of growth in a humidified atmosphere: 37°C, 5% CO₂, with 1 μ Ci of tritiated thymidine (Amersham, Arlington Heights, IL, at

1 mCi/ml) was added to each well. After an additional 24 hours of growth, the cells were transferred to glass tubes using 20 μ l of 100 mM EDTA dissolved in MEM media with 10% fetal bovine serum. After washing the wells with an additional 200 μ l of MEM
5 with 10% fetal bovine serum, 500 μ l of 10% trichloroacetic acid (TCA) was added to each glass tube in order to precipitate the incorporated tritiated thymidine. The TCA/cell mixture was allowed to incubate on ice for 20 minutes prior to filtration onto glass filters (Schleicher and Schuell, Keene, NH). The
10 filters were further washed with 5% TCA and 100% ethanol prior to drying and counting by scintillation. Concanavalin A (1 μ g/ml to 100 μ g/ml, as indicated) was used as a positive control for mitogenic activity.

15 The results are shown in Figure 9 for E. coli DNase I, the heparin-sepharose fraction of Example 3, purified Fractions I and II of Example 3, and the recombinant S. pyogenes DNase B of Example 3. The results indicate that both the purified Fractions I and II, as well as the recombinant DNase B, are
20 substantially free of mitogenic activity. The heparin-sepharose fraction did have detectable mitogenic activity, which was removed by further purification. This indicates that any mitogenic activity resided not in the DNase B protein, but in one or more contaminants.

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Example 12

Construction of a Vector Which Expresses DNase B in E. coli Which is Processed Identically to the Native DNase B

30

The genetic construction described in Examples 5 and 6 produced a recombinant DNase B protein in E. coli which differed from the natural DNase B protein in that the protein was processed at an amino acid one amino acid up from the

natural processing site. In order to produce a recombinant DNase B in E. coli which was absolutely identical to the fully processed and natural DNase B, a genetic construction was made which deletes this "extra" arginine at amino acid 43 (Ala-Arg-Gln-Thr-Gln-Val). This construction was made by using the polymerase chain reaction (PCR) to incorporate modified ends to the DNAase B gene in the λ 2-6 clone. The following oligonucleotides were designed and synthesized on the Pharmacia Gene Assembler Plus DNA synthesizer following the manufacturer's recommendations:

5 A. 5'-A-G-G-C-A-A-T-G-G-A-T-C-C-G-A-A-C-C-T-G-C-T-
G-G-G-T-T-C-C-C-G-T-C-G-T-G-T-T-T-T-C-T-C-C-A-A-A-A-A-T-G-C-
C-G-T-C-T-G-G-T-T-A-A-A-T-T-C-T-C-C-A-T-G-G-T-T-G-C-T-C-T-G-G-
T-T-T-C-C-G-C-T-A-C-C-A-T-G-G-C-T-G-T-T-A-C-C-A-C-C-G-T-T-A-C-
C-C-T-G-G-A-A-A-A-C-A-C-C-G-C-T-C-T-G-G-C-T-C-A-G-A-C-A-C-A-G-
G-T-C-T-C-A-A-A-T-G-A-T-G-T-T-G-T-T-C-T-A-A-A-T-G-A-T-G-G-C-G-
C-A-A-G-C-3' (SEQ ID NO: 12)

10 B. 3'-T-C-T-T-T-T-C-G-T-T-A-C-T-A-A-C-G-G-C-A-G-T-A-
A-C-G-G-G-G-C-C-C-A-G-C-T-G-G-G-C-C-5' (SEQ ID NO: 13)

15 These oligonucleotides were used as primers in a PCR
reaction using the AmpliTaq kit (Perkin-Elmer-Cetus, Norwalk,
CT), according to the manufacturer's instructions. The final
concentration of MgCl₂ was adjusted to 4 mM, and a 20 cycle
reaction was performed (37°C, 2 minutes; 72°C, 3 minutes;
95°C, 2 minutes) using the Perkin-Elmer 480 thermal cycler.
DNA of the λ gt11 clone 2-6 (100 ng) was used as a template
20 along with 200 μM of each primer. The resulting amplified
product was further digested with Bam HI and Sal I prior to
insertion into the Δ 33 expression vector. These
manipulations created a translational fusion with the sequence
as shown in Figure 10 (SEQ ID NO: 14), which is regulated by
25 the λ pL promoter.

C 600 Cl+, gal K- bacteria were transformed with the
ligation mixture and plated on to LB- Amp plates. Thereafter,

a minipreparation of DNA was made (F.M. Ausubel et al., eds., "Current Protocols in Molecular Biology" (John Wiley, 1987), Section 1.6), followed by cutting the plasmid with the enzymes Bam HI and Sal I to determine if the plasmid comprised the recombinant DNase B fragment. Plasmids of the desired construction were further transformed into the AR 120 host strain. These host cells with plasmids comprising the recombinant DNase B were then subjected to induction via the nalidixic acid protocol (Mott et al., supra). Colonies comprising the transformed AR120 were lifted from the agar plates and inoculated in Superbroth (base: 12 g yeast extract, 5 ml glycerol, 900 ml distilled H₂O; salts per liter of base: 1.7 g KH₂PO₄ and 15.8 g K₂HPO₄ anhydrous in 100 ml of deionized H₂O plus 100 µg/ml ampicillin) and grown at 37°C until the optical density of the culture at 600 nm equalled 0.4

Thereafter, nalidixic acid was added to the inoculated mixture at a final concentration of 60 µg/ml. The culture was incubated at 37°C for about 8 hours or, alternatively, overnight (approximately 16 hours). All cell fractions were assayed for DNase B activity including supernatant from the culture, sonicated cell pellets, and supernatants from the sonicated cell pellets. For the overnight induction, DNase B was secreted outside the E. coli cell. Quantities of DNase B were enough to be visualized by Coomassie stain, on polyacrylamide gel electrophoresis (PAGE). An IEF (3-9) gel was run, and silver stained. Compared to the previous clone under the pL promotor, there was a clear shift

of the pI to the more acidic side.

Example 13

5

Purification of the Arginine Deletion Construction and Amino Acid Sequence Analysis

Growth and purification of the recombinant DNase B
10 clone described in Example 12 was performed as described in
Example 6. The amino acid sequence was determined using a
Beckman Microsequencing System 2020/Gold. The amino acid
sequence was determined to be Q-T-Q-V-S-N-D-V-V-L-N-D-G-A-S-K-
Y-L-N-E-A-L-A-W-T-F-N-D-S-P-N-Y (SEQ ID NO: 16). This
15 sequence analysis demonstrates that the cleavage product of
the arginine deleted construction is identical to the natural
DNase B protein produced in Streptococcus pyogenes.

20

Example 14

Coupling of DNase B to Carboxylate Latex Particles

(Prospective Example)

25

Carboxylate latex particles (Interfacial Dynamics
Corp.) are coupled to DNase B. The particles are described as
high density carboxylate latex particles. The particles

typically come in approximately 4% solids in surfactant-free distilled water. Both the 29 nm and the 17 nm diameter particles can be used.

5 Latex particles (5 ml) are diluted 2X with 0.1 M sodium bicarbonate buffer, pH 7.75. On ice, with stirring, the carboxyl groups of the latex particles are activated using a water-soluble carbodiimide, 1-ethyl-3-(3-
10 dimethylaminopropyl) carbodiimide (EDAC). A 20 mg/ml EDAC solution is made and added to the latex particles to a final concentration of 10 mM with constant stirring. After the addition of EDAC, N-hydroxysuccinimide (NHS) (80 mg/ml
15 solution) is added to a final concentration of 70 mM. The latex particle solution turns turbid after the coupling reagents are added. The solution is then stirred for 1 hour on ice.

 The excess reagents are removed by centrifugation in a Beckman J2-21M, JA 20 rotor at 18,000 rpm for 30 minutes at
20 10°C. The supernatant is decanted. The latex particles are resuspended in 10 ml of phosphate buffered saline (PBS) containing 1% Tween-20 and 5 mg/ml recombinant DNase B. Small clumps are dispersed with gentle sonication for approximately 1-2 minutes with a Heat Systems-Ultrasonics Inc. sonicator,
25 model W-385. The setting is at 2 using a microtip. The reaction mixture is slightly agitated at room temperature from approximately 20 hours.

The unbound DNase B is removed by ultracentrifugation in a Beckman L8-70, Ti60 rotor, at 30,000 rpm for 30 minutes. The supernatant is removed. The latex particles are resuspended in 10 ml of PBS. Small clumps are dispersed by sonication at the same setting as above for approximately 1-2 minutes. The PBS is removed by ultracentrifugation as described above. The washed latex particles are resuspended in 10 mL PBS + 0.01% sodium azide. Alternatively, unbound DNase B can be removed by size exclusion chromatography. The final DNase B latex particle stock (approximately 2%) is stored at 4°C until further dilution for use in an assay.

ADVANTAGES OF THE INVENTION

The present invention provides a method of obtaining highly purified S. pyogenes DNase B enzyme without the necessity of growing large quantities of S. pyogenes, an expensive and risky process. The enzyme can be obtained without having to purify it from other proteins of S. pyogenes; rather, the enzyme can be purified from recombinant phage-infected Escherichia coli or from E. coli transfected with an appropriate expression vector. The expression vector can be chosen so as to optimize expression.

The S. pyogenes DNase B can then be used to assay for anti-DNase B antibody in serum in an assay specific for

DNase B. In particular, the availability of purified DNase B makes possible the use of an ELISA assay using purified enzyme adsorbed to the solid phase, which is an assay suitable for wide use and easy and convenient to perform. The assay is also of high sensitivity and specificity. Such an assay is particularly suitable for clinical use in detecting S. pyogenes infection.

Although the present invention has been described in considerable detail with regard to certain preferred versions thereof, other versions are possible. Therefore, the spirit and scope of the appended claims should not be limited to the description of the preferred version contained herein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Beckman Instruments, Inc.
2500 Harbor Boulevard
Fullerton, California 92634

(ii) TITLE OF INVENTION: Recombinant DNase B Derived from
Streptococcus pyogenes

(iii) NUMBER OF SEQUENCES: 16

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Sheldon & Mak
(B) STREET: 225 South Lake Avenue, Ninth Floor
(C) CITY: Pasadena
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 91001

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/082,845
- (B) FILING DATE:
- (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Farber, Michael B.
- (B) REGISTRATION NUMBER: 32,612
- (C) REFERENCE/DOCKET NUMBER: 9521

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (818) 796-4000
- (B) TELEFAX: (818) 795-6321

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Streptococcus pyogenes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Asn Leu Leu Gly Ser Arg Arg Val Phe Ser Lys Lys Cys Arg Leu
1 5 10 15

Val Lys Phe Ser Met Val Ala Leu Val Ser Ala Thr Met Ala Val Thr
 20 25 30

Thr Val Thr Leu Glu Asn Thr Ala Leu Ala Arg
 35 40

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Synthetic DNA primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TAACGGATCC GAATCTACTT GGATCAAGAC GGGTTTTTTTC T

41

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Synthetic DNA primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCGGGTCGAC CCGGGGAATG ACGGCAATCA TTGCTTTTTC T

41

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Streptococcus pyogenes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gln Thr Gln Val Ser Asn Asp Val Val Leu Asn Asp Gly Ala Ser Xaa

1

5

10

15

Tyr Leu Asn Glu Ala Leu Ala

20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Synthetic probe

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAUACNCART NWSNAAYGAY GT

22

(2) INFORMATION FOR SEQ ID NO. 6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1083 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Streptococcus pyogenes

- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 129..944

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GACAACGCCT TCTTTTTTCT CTTACTATC TCCTTTAATT TTCATATTTT TAAAAAAAC 60

TATTGATAAA CTAGTTAAGT AAGCGTATAC TATGGTTAGT TAGCGAAATT AGAAAAGAGG 120

ACAAGCAT ATG AAT CTA CTT GGA TCA AGA CGG GTT TTT TCT AAA AAA TGT	170
Met Asn Leu Leu Gly Ser Arg Arg Val Phe Ser Lys Lys Cys	
1 5 10	
CGG CTA GTA AAA TTT TCA ATG GTA GCT CTT GTA TCA GCC ACA ATG GCT	218
Arg Leu Val Lys Phe Ser Met Val Ala Leu Val Ser Ala Thr Met Ala	
15 20 25 30	
GTA ACA ACA GTC ACA CTT GAA AAT ACT GCA CTG GCA CGA CAA ACA CAG	266
Val Thr Thr Val Thr Leu Glu Asn Thr Ala Leu Ala Arg Gln Thr Gln	
35 40 45	
GTC TCA AAT GAT GTT GTT CTA AAT GAT GGC GCA AGC AAG TAC CTA AAC	314
Val Ser Asn Asp Val Val Leu Asn Asp Gly Ala Ser Lys Tyr Leu Asn	
50 55 60	
GAA GCA TTA GCT TGG ACA TTC AAT GAC AGT CCT AAC TAT TAC AAA ACT	362
Glu Ala Leu Ala Trp Thr Phe Asn Asp Ser Pro Asn Tyr Tyr Lys Thr	
65 70 75	
TTA GGT ACT AGT CAG ATT ACT CCA GCA CTC TTT CCT AAA GCA GGA GAT	410
Leu Gly Thr Ser Gln Ile Thr Pro Ala Leu Phe Pro Lys Ala Gly Asp	
80 85 90	
ATT CTC TAT AGC AAA TTA GAT GAG TTA GGA AGG ACG CGT ACT GCT AGA	458
Ile Leu Tyr Ser Lys Leu Asp Glu Leu Gly Arg Thr Arg Thr Ala Arg	
95 100 105 110	

CCA ATC TAC AAC GCA GAC GAG TTG ATT CCA AGA GCT GTC GTG GTA TCA	842
Pro Ile Tyr Asn Ala Asp Glu Leu Ile Pro Arg Ala Val Val Val Ser	
225 230 235	
ATG CAA TCT TCT GAT AAT ACC ATC AAC GAG AAA GTA TTA GTT TAC AAC	890
Met Gln Ser Ser Asp Asn Thr Ile Asn Glu Lys Val Leu Val Tyr Asn	
240 245 250	
ACA GCT AAT GGC TAC ACC ATT AAC TAC CAT AAC GGT ACA CCT ACT CAA	938
Thr Ala Asn Gly Tyr Thr Ile Asn Tyr His Asn Gly Thr Pro Thr Gln	
255 260 265 270	
AAA TAATACCAAA AGGCTAGACC TCTGCTCACT AGGCCTAGCT TTTTACATCA	991
Lys	
AAAAAAGCAA TGA CTATAGA AAGTAAAAAT ACTAGAAAAA GCAATGATTG CCGTCATTGC	1051
TTTTTATGAA TTTGTGCAAA AAGCAAAAAA GC	1083

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 271 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

Gly Lys Asn Gln Asn Pro Ala Gly Trp Thr Gly Asn Pro Asn His Val
 130 135 140

Lys Tyr Lys Ile Glu Trp Leu Asn Gly Leu Ser Tyr Val Gly Asp Phe
 145 150 155 160

Trp Asn Arg Ser His Leu Ile Ala Asp Ser Leu Gly Gly Asp Ala Leu
 165 170 175

Arg Val Asn Ala Val Thr Gly Thr Arg Thr Gln Asn Val Gly Gly Arg
 180 185 190

Asp Gln Lys Gly Gly Met Arg Tyr Thr Glu Gln Arg Ala Gln Glu Trp
 195 200 205

Leu Glu Ala Asn Arg Asp Gly Tyr Leu Tyr Tyr Glu Val Ala Pro Ile
 210 215 220

Tyr Asn Ala Asp Glu Leu Ile Pro Arg Ala Val Val Val Ser Met Gln
 225 230 235 240

Ser Ser Asp Asn Thr Ile Asn Glu Lys Val Leu Val Tyr Asn Thr Ala
 245 250 255

Asn Gly Tyr Thr Ile Asn Tyr His Asn Gly Thr Pro Thr Gln Lys
 260 265 270

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 229 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Streptococcus pyogenes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Arg Gln Thr Gln Val Ser Asn Asp Val Val Leu Asn Asp Gly Ala Ser
 1 5 10 15

Lys Tyr Leu Asn Glu Ala Leu Ala Trp Thr Phe Asn Asp Ser Pro Asn
 20 25 30

Tyr Tyr Lys Thr Leu Gly Thr Ser Gln Ile Thr Pro Ala Leu Phe Pro
 35 40 45

Lys Ala Gly Asp Ile Leu Tyr Ser Lys Leu Asp Glu Leu Gly Arg Thr
 50 55 60

Arg Thr Ala Arg Gly Thr Leu Thr Tyr Ala Asn Val Glu Gly Ser Tyr
 65 70 75 80

Gly Val Arg Gln Ser Phe Gly Lys Asn Gln Asn Pro Ala Gly Trp Thr
 85 90 95

Gly Asn Pro Asn His Val Lys Tyr Lys Ile Glu Trp Leu Asn Gly Leu
 100 105 110

Ser Tyr Val Gly Asp Phe Trp Asn Arg Ser His Leu Ile Ala Asp Ser
 115 120 125

Leu Gly Gly Asp Ala Leu Arg Val Asn Ala Val Thr Gly Thr Arg Thr
 130 135 140

Gln Asn Val Gly Gly Arg Asp Gln Lys Gly Gly Met Arg Tyr Thr Glu
 145 150 155 160

Gln Arg Ala Gln Glu Trp Leu Glu Ala Asn Arg Asp Gly Tyr Leu Tyr
 165 170 175

Tyr Glu Val Ala Pro Ile Tyr Asn Ala Asp Glu Leu Ile Pro Arg Ala
 180 185 190

Val Val Val Ser Met Gln Ser Ser Asp Asn Thr Ile Asn Glu Lys Val
195 200 205

Leu Val Tyr Asn Thr Ala Asn Gly Tyr Thr Ile Asn Tyr His Asn Gly
210 215 220

Thr Pro Thr Gln Lys
225

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 200 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Streptococcus pyogenes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
GACAACGCCT TCTTTTTTCT CCTTACTATC TCCTTTAATT TTCATATTTT TTAAAAAAC    60
TATTGATAAA CTAGTTAAGT AAGCGTATAC TATGGTTAGT TAGCGAAATT AGAAAAGAGG    120
ACAAGCATAT GAATCTACTT GGATCAAGAC GGGTTTTTTC TAAAAAATGT CGGCTAGTAA    180
AATTTTCAAT GGTAGCTCTT                                                    200
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 940 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Streptococcus pyogenes*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATGGATCCGA ATCTACTTGG ATCAAGACGG GTTTTTTCTA AAAAATGTCG GCTAGTAAAA	60
TTTTCAATGG TAGCTCTTGT ATCAGCCACA ATGGCTGTAA CAACAGTCAC ACTTGAAAAT	120
ACTGCACTGG CACGACAAAC ACAGGTCTCA AATGATGTTG TTCTAAATGA TGGCGCAAGC	180
AAGTACCTAA ACGAAGCATT AGCTTGGACA TTCAATGACA GTCCTAACTA TTACAAAAT	240
TTAGGTACTA GTCAGATTAC TCCAGCACTC TTTCTAAAG CAGGAGATAT TCTCTATAGC	300
AAATTAGATG AGTTAGGAAG GACGCGTACT GCTAGAGGTA CATTGACTTA TGCCAATGTT	360
GAAGGTAGCT ACGGTGTTAG ACAATCTTTC GGTA AAAATC AAAACCCCGC AGGATGGACT	420
GGAAACCCTA ATCATGTCAA ATATAAAAT GAATGGTTAA ATGGTCTATC TTATGTCGGA	480
GATTTCTGGA ATAGAAGTCA TCTCATTGCA GATAGTCTCG GTGGAGATGC ACTCAGAGTC	540
AATGCCGTTA CAGGAACACG TACCCAAAAT GTAGGAGGTC GTGACCAAAA AGGCGGCATG	600
CGCTATACCG AACAAAGAGC TCAAGAAATG TTAGAAGCAA ATCGTGATGG CTATCTTTAT	660
TATGAAGTCG CTCCAATCTA CAACGCAGAC GAGTTGATTC CAAGAGCTGT CGTGGTATCA	720
ATGCAATCTT CTGATAATAC CATCAACGAG AAAGTATTAG TTTACAACAC AGCTAATGGC	780

TACACCATTA ACTACCATAA CGGTACACCT ACTCAAAAAT AATACCAAAA GGCTAGACCT 840

CTGCTCACTA GGCCTAGCTT TTTACATCAA AAAAAGCAAT GACTATAGAA AGTAAAAATA 900

CTAGAAAAAG CAATGATTGC CGTCATTGCC CCGGGTCGAC 940

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 182 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Synthetic primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGGCAATGGA TCCGAACCTG CTGGGTCCG GTCGTGTTTT CTCAAAAAA TGCCGTCTGG 60

TTAAATTCTC CATGGTTGCT CTGGTTTCCG CTACCATGGC TGTTACCACC GTTACCCTGG

AAAACACCGC TCTGGCTCAG ACACAGGTCT CAAATGATGT TGTTCTAAAT GATGGCGCAA

GC

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Synthetic primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCGGGTCGAC CCGGGGCAAT GACGGCAATC ATTGCTTTTC T

CGT CTG GTT AAA TTC TCC ATG GTT GCT CTG GTT TCC GCT ACC ATG GCT	96
Arg Leu Val Lys Phe Ser Met Val Ala Leu Val Ser Ala Thr Met Ala	
20 25 30	
GTT ACC ACC GTT ACC CTG GAA AAC ACC GCT CTG GCT CAG ACA CAG GTC	144
Val Thr Thr Val Thr Leu Glu Asn Thr Ala Leu Ala Gln Thr Gln Val	
35 40 45	
TCA AAT GAT GTT GTT CTA AAT GAT GGC GCA AGC AAG TAC CTA AAC GAA	192
Ser Asn Asp Val Val Leu Asn Asp Gly Ala Ser Lys Tyr Leu Asn Glu	
50 55 60	
GCA TTA GCT TGG ACA TTC AAT GAC AGT CCT AAC TAT TAC AAA ACT TTA	240
Ala Leu Ala Trp Thr Phe Asn Asp Ser Pro Asn Tyr Tyr Lys Thr Leu	
65 70 75 80	
GGT ACT AGT CAG ATT ACT CCA GCA CTC TTT CCT AAA GCA GGA GAT ATT	288
Gly Thr Ser Gln Ile Thr Pro Ala Leu Phe Pro Lys Ala Gly Asp Ile	
85 90 95	
CTC TAT AGC AAA TTA GAT GAG TTA GGA AGG ACG CGT ACT GCT AGA GGT	336
Leu Tyr Ser Lys Leu Asp Glu Leu Gly Arg Thr Arg Thr Ala Arg Gly	
100 105 110	
ACA TTG ACT TAT GCC AAT GTT GAA GGT AGC TAC GGT GTT AGA CAA TCT	384
Thr Leu Thr Tyr Ala Asn Val Glu Gly Ser Tyr Gly Val Arg Gln Ser	
115 120 125	

TTC GGT AAA AAT CAA AAC CCC GCA GGA TGG ACT GGA AAC CCT AAT CAT	432
Phe Gly Lys Asn Gln Asn Pro Ala Gly Trp Thr Gly Asn Pro Asn His	
130 135 140	
GTC AAA TAT AAA ATT GAA TGG TTA AAT GGT CTA TCT TAT GTC GGA GAT	480
Val Lys Tyr Lys Ile Glu Trp Leu Asn Gly Leu Ser Tyr Val Gly Asp	
145 150 155 160	
TTC TGG AAT AGA AGT CAT CTC ATT GCA GAT AGT CTC GGT GGA GAT GCA	528
Phe Trp Asn Arg Ser His Leu Ile Ala Asp Ser Leu Gly Gly Asp Ala	
165 170 175	
CTC AGA GTC AAT GCC GTT ACA GGA ACA CGT ACC CAA AAT GTA GGA GGT	576
Leu Arg Val Asn Ala Val Thr Gly Thr Arg Thr Gln Asn Val Gly Gly	
180 185 190	
CGT GAC CAA AAA GGC GGC ATG CGC TAT ACC GAA CAA AGA GCT CAA GAA	624
Arg Asp Gln Lys Gly Gly Met Arg Tyr Thr Glu Gln Arg Ala Gln Glu	
195 200 205	
TGG TTA GAA GCA AAT CGT GAT GGC TAT CTT TAT TAT GAA GTC GCT CCA	672
Trp Leu Glu Ala Asn Arg Asp Gly Tyr Leu Tyr Tyr Glu Val Ala Pro	
210 215 220	
ATC TAC AAC GCA GAC GAG TTG ATT CCA AGA GCT GTC GTG GTA TCA ATG	720
Ile Tyr Asn Ala Asp Glu Leu Ile Pro Arg Ala Val Val Val Ser Met	
225 230 235 240	

Met Asp Pro Asn Leu Leu Gly Ser Arg Arg Val Phe Ser Lys Lys Cys
 1 5 10 15
 Arg Leu Val Lys Phe Ser Met Val Ala Leu Val Ser Ala Thr Met Ala
 20 25 30
 Val Thr Thr Val Thr Leu Glu Asn Thr Ala Leu Ala Gln Thr Gln Val
 35 40 45
 Ser Asn Asp Val Val Leu Asn Asp Gly Ala Ser Lys Tyr Leu Asn Glu
 50 55 60
 Ala Leu Ala Trp Thr Phe Asn Asp Ser Pro Asn Tyr Tyr Lys Thr Leu
 65 70 75 80
 Gly Thr Ser Gln Ile Thr Pro Ala Leu Phe Pro Lys Ala Gly Asp Ile
 85 90 95
 Leu Tyr Ser Lys Leu Asp Glu Leu Gly Arg Thr Arg Thr Ala Arg Gly
 100 105 110
 Thr Leu Thr Tyr Ala Asn Val Glu Gly Ser Tyr Gly Val Arg Gln Ser
 115 120 125
 Phe Gly Lys Asn Gln Asn Pro Ala Gly Trp Thr Gly Asn Pro Asn His
 130 135 140

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Streptococcus pyogenes*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Gln	Thr	Gln	Val	Ser	Asn	Asp	Val	Val	Leu	Asn	Asp	Gly	Ala	Ser	Lys
1				5					10					15	
Tyr	Leu	Asn	Glu	Ala	Leu	Ala	Trp	Thr	Phe	Asn	Asp	Ser	Pro	Asn	Tyr
				20					25					30	

We claim:

5 1. Substantially purified DNA comprising DNA encoding an amino acid sequence selected from the group consisting of the amino acid sequence of: (i) Streptococcus pyogenes DNase B enzyme as shown in Figure 4; and (ii) a sequence encoding a functional equivalent of S. pyogenes DNase B enzyme, the DNA being substantially free of DNA that does not encode the amino acid sequence of Figure 4 or a functional equivalent of S. pyogenes DNase B enzyme except for a leader peptide fused to the amino terminus of S. pyogenes DNase B enzyme.

15 2. The DNA of claim 1 wherein the DNA further comprises a DNA sequence encoding a leader peptide fused to the amino terminus of S. pyogenes DNase B enzyme.

20 3. The DNA of claim 1 having the nucleotide sequence of Figure 3.

25 4. An expression vector for Streptococcus pyogenes DNase B enzyme comprising the DNA sequence of claim 1 operatively linked to at least one control sequence compatible with a suitable bacterial host cell.

30 5. An expression vector for Streptococcus pyogenes DNase B enzyme comprising the DNA sequence of claim 3 operatively linked to at least one control sequence compatible with a suitable bacterial host cell.

6. The vector of claim 4 wherein the DNA encoding the Streptococcus pyogenes DNase B enzyme is linked to at least one sequence from bacteriophage λ .

35 7. The vector of claim 5 wherein the DNA encoding the Streptococcus pyogenes DNase B enzyme is linked to at least one sequence from bacteriophage λ .

8. A bacterial host cell transformed with the expression vector of claim 4 in a manner allowing the transformed bacterial host cell to express the Streptococcus pyogenes DNase B encoded by the DNA incorporated within the expression vector of claim 4 in a detectable quantity.

9. A bacterial host cell transformed with the expression vector of claim 5 in a manner allowing the transformed bacterial host cell to express the Streptococcus pyogenes DNase B encoded by the DNA incorporated within the expression vector of claim 5 in a detectable quantity.

10. Substantially purified S. pyogenes DNase B enzyme comprising a protein having the amino acid sequence of Figure 4.

11. A process for producing substantially purified Streptococcus pyogenes DNase B enzyme comprising:

- (a) culturing the bacterial host cell of claim 8;
- (b) using the cultured bacterial host cell to express the DNase B enzyme; and
- (c) purifying the enzyme from the cultured bacterial host cell.

12. A process for producing substantially purified Streptococcus pyogenes DNase B enzyme comprising:

- (a) culturing the bacterial host cell of claim 9;
- (b) using the cultured bacterial host cell to express the DNase B enzyme; and
- (c) purifying the enzyme from the cultured bacterial host cell.

13. Streptococcus pyogenes DNase B enzyme prepared by the process of claim 11.

14. Streptococcus pyogenes DNase B enzyme prepared by the process of claim 12.

15. Streptococcus pyogenes DNase B enzyme fused at its amino terminus with a leader peptide, the leader peptide having the sequence M-N-L-L-G-S-R-R-V-F-S-K-K-C-R-L-V-K-F-S-M-V-A-L-V-S-A-T-M-A-V-T-T-V-T-L-E-N-T-A-L-A-R (SEQ ID NO: 1).

5

16. A mutant of the protein whose amino acid sequence is shown in Figure 4 in which at least one of the following mutations occurs:

(a) a deletion of one or more amino acids from the sequence of Figure 4;

10

(b) an insertion of one or more naturally-occurring L-amino acids into the sequence of Figure 4; and

(c) replacement of at least one of the amino acids of Figure 4 with an alternative naturally occurring L-amino acid;

15

the resulting mutant having reduced or increased DNase B activity or another altered property.

17. The mutant protein of claim 16 wherein the mutant substantially maintains the antigenic reactivity of natural S. pyogenes DNase B enzyme.

20

18. A transcriptional fusion comprising at least a portion of the S. pyogenes DNase B DNA sequence of claim 3 fused with another gene, with the fusion having a detectable property altered from the property of the sequence of claim 3.

25

19. A translational fusion comprising at least a portion of the protein coded for by the S. pyogenes DNase B protein sequence of claim 3 fused with another protein, with the fusion having a detectable property altered from the property of the protein coded for by the sequence of claim 3.

30

20. Substantially purified Streptococcus pyogenes DNase B enzyme substantially free of proteins other than: (1) the Streptococcus DNase B enzyme and (2) Streptococcus DNase B

35

enzyme fused at its amino terminus with a leader peptide, the substantially purified protein being substantially free of mitogenic activity.

5 21. The substantially purified S. pyogenes DNase B enzyme of claim 20 comprising Fraction I of S. pyogenes DNase B enzyme and substantially free of Fraction II of S. pyogenes DNase B enzyme.

10 22. The substantially purified S. pyogenes DNase B enzyme of claim 20 comprising Fraction II of S. pyogenes DNase B enzyme and substantially free of Fraction I of S. pyogenes DNase B enzyme.

15 23. A process for preparing substantially purified Streptococcus pyogenes DNase B enzyme comprising:

- (a) absorption to and elution from diethylaminoethyl cellulose to produce a first eluate;
- 20 (b) chromatography of the first eluate on phenyl agarose to produce a second eluate;
- (c) chromatography of the second eluate on heparin agarose to produce a third eluate; and
- (d) chromatofocusing of the third eluate to produce substantially purified DNase B enzyme.

25 24. The process of claim 23 further comprising purification of the substantially purified DNase B by reverse-phase high-pressure liquid chromatography.

30 25. Substantially purified Streptococcus pyogenes DNase B enzyme produced by the process of claim 23.

 26. A single-stranded nucleic acid probe hybridizing with the DNA sequence coding for the amino-terminal 23 amino acids of the Streptococcus pyogenes DNase B enzyme, not including any portion of the leader sequence thereof, with no greater than about a 30% mismatch.

35

27. An antibody specifically binding the Streptococcus pyogenes DNase B enzyme of claim 13.

5 28. An antibody specifically binding the Streptococcus pyogenes DNase B enzyme of claim 14.

29. An antibody specifically binding the Streptococcus pyogenes DNase B enzyme of claim 20.

10 30. An antibody specifically binding the Streptococcus pyogenes DNase B enzyme of claim 25.

15 31. A monoclonal antibody specifically binding the Streptococcus pyogenes DNase B enzyme of claim 13.

32. A monoclonal antibody specifically binding the Streptococcus pyogenes DNase B enzyme of claim 14.

20 33. A monoclonal antibody specifically binding the Streptococcus pyogenes DNase B enzyme of claim 20.

34. A monoclonal antibody specifically binding the Streptococcus pyogenes DNase B enzyme of claim 25.

25 35. A method for detecting and/or determining anti-Streptococcus pyogenes DNase B antibody in a test sample, comprising the steps of:

(a) providing a test sample suspected of containing anti-Streptococcus pyogenes DNase B antibody;

30 (b) adding a quantity of the Streptococcus pyogenes DNase B enzyme of claim 13 to the test sample, the quantity being sufficient to produce a detectable level of enzymatic activity in the absence of inhibition of the enzymatic activity by anti-DNase B antibody in the test sample; and

35 (c) determining a level of activity of DNase B enzyme in the test sample by performing an enzyme assay to detect and/or determine the anti-Streptococcus pyogenes

antibody in the test sample.

36. A method for detecting and/or determining anti-Streptococcus pyogenes DNase B antibody in a test sample, comprising the steps of:

(a) providing a test sample suspected of containing anti-Streptococcus pyogenes DNase B antibody;

(b) adding a quantity of the Streptococcus pyogenes DNase B enzyme of claim 14 to the test sample, the quantity being sufficient to produce a detectable level of enzymatic activity in the absence of inhibition of the enzymatic activity by anti-DNase B antibody in the test sample; and

(c) determining the level of antibody of DNase B enzyme in the test sample to detect and/or determine the anti-Streptococcus pyogenes antibody in the test sample.

37. A method for detecting and/or determining anti-Streptococcus pyogenes DNase B antibody in a test sample, comprising the steps of:

(a) providing a test sample suspected of containing anti-Streptococcus pyogenes DNase B antibody;

(b) adding a quantity of the Streptococcus pyogenes DNase B enzyme of claim 20 to the test sample, the quantity being sufficient to produce a detectable level of enzymatic activity in the absence of inhibition of the enzymatic activity by anti-DNase B antibody in the test sample; and

(c) determining the level of activity of DNase B enzyme in the test sample by performing an enzyme assay to detect and/or determine anti-Streptococcus pyogenes antibody in the test sample.

38. A method for detecting and/or determining anti-Streptococcus pyogenes DNase B antibody in a test sample, comprising the steps of:

(a) providing a test sample suspected of containing anti-Streptococcus pyogenes DNase B antibody;

(b) adding a quantity of the Streptococcus pyogenes

DNase B enzyme of claim 25 to the test sample, the quantity being sufficient to produce a detectable level of enzymatic activity in the absence of inhibition of the enzymatic activity by anti-DNase B antibody in the test sample; and

5 (c) determining the level of activity of DNase B enzyme in the test sample by performing an enzyme assay to detect and/or determine anti-Streptococcus pyogenes antibody in the test sample.

10 39. A method for detecting and/or determining anti-Streptococcus pyogenes DNase B antibody in a test sample, comprising the steps of:

(a) binding the Streptococcus pyogenes DNase B enzyme of claim 13 to a solid support;

15 (b) reacting a test sample suspected of containing anti-Streptococcus pyogenes DNase B antibody with the Streptococcus pyogenes DNase B enzyme bound to the solid support to bind the antibody to the enzyme and thus to the solid support; and

20 (c) detecting the antibody bound to the solid support to detect and/or determine the antibody in the test sample.

25 40. A method for detecting and/or determining anti-Streptococcus pyogenes DNase B antibody in a test sample, comprising the steps of:

(a) binding the Streptococcus pyogenes DNase B enzyme of claim 14 to a solid support;

30 (b) reacting a test sample suspected of containing anti-Streptococcus pyogenes DNase B antibody with the Streptococcus pyogenes DNase B enzyme bound to the solid support to bind the antibody to the enzyme and thus to the solid support; and

35 (c) detecting the antibody bound to the solid support to detect and/or determine the antibody in the test sample.

41. A method for detecting and/or determining anti-Streptococcus pyogenes DNase B antibody in a test sample, comprising the steps of:

5 (a) binding the Streptococcus pyogenes DNase B enzyme of claim 20 to a solid support;

(b) reacting a test sample suspected of containing anti-Streptococcus pyogenes DNase B antibody with the Streptococcus pyogenes DNase B enzyme bound to the solid support to bind the antibody to the enzyme and thus to the
10 solid support; and

(c) detecting the antibody bound to the solid support to detect and/or determine the antibody in the test sample.

15 42. A method for detecting and/or determining anti-Streptococcus pyogenes DNase B antibody in a test sample, comprising the steps of:

(a) binding the Streptococcus pyogenes DNase B enzyme of claim 25 to a solid support;

20 (b) reacting a test sample suspected of containing anti-Streptococcus pyogenes DNase B antibody with the Streptococcus pyogenes DNase B enzyme bound to the solid support to bind the antibody to the enzyme and thus to the solid support; and

25 (c) detecting the antibody bound to the solid support to detect and/or determine the antibody in the test sample.

30 43. A method for detecting and/or determining anti-Streptococcus pyogenes DNase B antibody in a test sample, comprising the steps of:

(a) preparing a buffered solution of the DNase B of claim 13;

35 (b) reacting the buffered DNase B solution with a test sample suspected of containing anti-S. pyogenes DNase B antibody; and

(c) detecting a reaction between the DNase B and the

anti-DNase B antibody by observing and/or measuring a change in light absorption and/or light scattering in the solution.

5 44. A method for detecting and/or determining anti-Streptococcus pyogenes DNase B antibody in a test sample, comprising the steps of:

(a) preparing a buffered solution of the DNase B of claim 14;

10 (b) reacting the buffered DNase B solution with a test sample suspected of containing anti-S. pyogenes DNase B antibody; and

(c) detecting a reaction between the DNase B and the anti-DNase B antibody by observing and/or measuring a change in light absorption and/or light scattering in the solution.

15

45. A method for detecting and/or determining anti-Streptococcus pyogenes DNase B antibody in a test sample, comprising the steps of:

20 (a) preparing a buffered solution of the DNase B of claim 20;

(b) reacting the buffered DNase B solution with a test sample suspected of containing anti-S. pyogenes DNase B antibody; and

25 (c) detecting a reaction between the DNase B and the anti-DNase B antibody by observing and/or measuring a change in light absorption and/or light scattering in the solution.

30 46. A method for detecting and/or determining anti-Streptococcus pyogenes DNase B antibody in a test sample, comprising the steps of:

(a) preparing a buffered solution of the DNase B of claim 25;

35 (b) reacting the buffered DNase B solution with a test sample suspected of containing anti-S. pyogenes DNase B antibody; and

(c) detecting a reaction between the DNase B and the anti-DNase B antibody by observing and/or measuring a change

in light absorption and/or light scattering in the solution.

47. A method of using a promoter originally associated with the S. pyogenes DNase B gene to express a protein other than DNase B comprising:

(a) separating the promoter originally associated with the S. pyogenes DNase B gene from the S. pyogenes DNase B gene;

(b) operatively linking the promoter with a structural gene for a S. pyogenes protein other than the gene for DNase B; and

(c) expressing the protein encoded by the structural gene.

48. The method of claim 47 wherein the protein is expressed in S. pyogenes.

49. The method of claim 48 wherein the protein is expressed in a prokaryote other than S. pyogenes.

50. A substantially purified promoter sequence derived from a promoter sequence originally associated with S. pyogenes DNase B including therein a start site for transcription and sites homologous to the consensus -10 and -35 sites of bacterial promoters.

51. A process for using a leader peptide associated with S. pyogenes DNase B enzyme to express a protein in a prokaryote comprises:

(1) fusing the DNA coding for the protein to DNA coding for a leader peptide, the leader peptide having the sequence M-N-L-L-G-S-R-R-V-F-S-K-K-C-R-L-V-K-F-S-M-V-A-L-V-S-A-T-M-A-V-T-T-V-T-L-E-N-T-A-L-A-R (SEQ ID NO: 1), so that the fused DNA codes for a recombinant protein with a single reading frame with the leader peptide being at the amino-terminus of the protein;

(2) introducing the fused DNA into the prokaryote;

and

(3) expressing the fused DNA in the prokaryote so that the recombinant protein is produced in a recoverable quantity.

5

52. The process of claim 51 wherein the prokaryote is E. coli.

10

53. The process of claim 51 wherein the prokaryote is a gram-positive bacterium selected from Staphylococcus, Streptococcus, and Streptomyces species.

15

54. The process of claim 51 wherein the recombinant protein is excreted into the culture medium of the prokaryote.

20

55. A method for immunizing a mammal against infection with S. pyogenes comprising administering a quantity of the purified S. pyogenes DNase B enzyme of claim 13 to the mammal sufficient to stimulate production of antibodies specific for S. pyogenes DNase B.

25

56. A method for immunizing a mammal against infection with S. pyogenes comprising administering a quantity of the purified S. pyogenes DNase B enzyme of claim 14 to the mammal sufficient to stimulate production of antibodies specific for S. pyogenes DNase B.

30

57. A method for immunizing a mammal against infection with S. pyogenes comprising administering a quantity of the purified S. pyogenes mutant DNase B enzyme of claim 17 to the mammal sufficient to stimulate production of antibodies specific for S. pyogenes DNase B.

35

58. A method for immunizing a mammal against infection with S. pyogenes comprising administering a quantity of the purified S. pyogenes DNase B enzyme of claim 20 to the mammal sufficient to stimulate production of antibodies

specific for S. pyogenes DNase B.

59. A method for immunizing a mammal against infection with S. pyogenes comprising administering a quantity
5 of the purified S. pyogenes DNase B enzyme of claim 25 to the mammal sufficient to stimulate production of antibodies specific for S. pyogenes DNase B.

60. A method for treating cystic fibrosis in a
10 patient with cystic fibrosis comprising:

- (a) generating an aerosol of the purified enzymatically active DNase B enzyme of claim 13; and
- (b) administering the aerosol to a patient with cystic fibrosis in a quantity sufficient to reduce lung fluid
15 viscosity in the patient.

61. A method for treating cystic fibrosis in a patient with cystic fibrosis comprising:

- (a) generating an aerosol of the purified
20 enzymatically active DNase B enzyme of claim 14; and
- (b) administering the aerosol to a patient with cystic fibrosis in a quantity sufficient to reduce lung fluid viscosity in the patient.

62. A method for treating cystic fibrosis in a patient with cystic fibrosis comprising:

- (a) generating an aerosol of the purified enzymatically active DNase B enzyme of claim 20; and
- (b) administering the aerosol to a patient with
30 cystic fibrosis in a quantity sufficient to reduce lung fluid viscosity in the patient.

63. A method for treating cystic fibrosis in a patient with cystic fibrosis comprising:

- (a) generating an aerosol of the purified
35 enzymatically active DNase B enzyme of claim 25; and
- (b) administering the aerosol to a patient with

cystic fibrosis in a quantity sufficient to reduce lung fluid viscosity in the patient.

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SUBCLONES OF DNaseB2 AND ITS ABILITY TO PRODUCE ACTIVE NUCLEASE

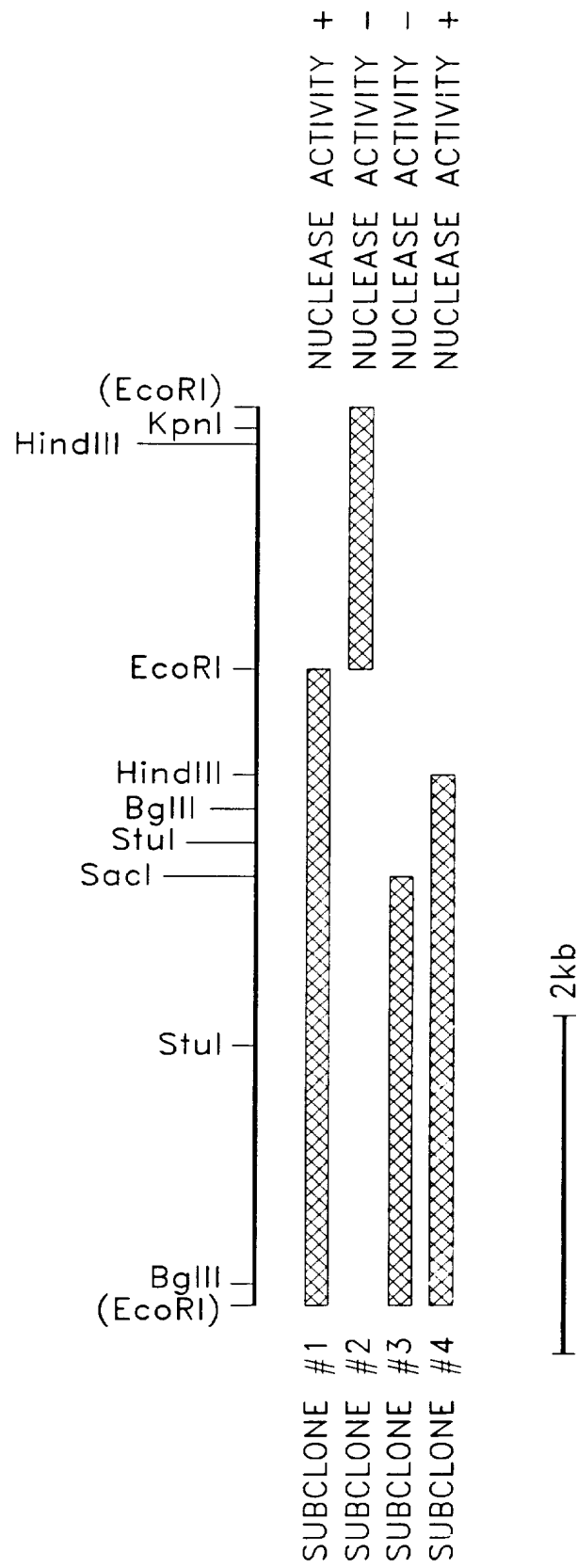


FIG. 2

5/11

AMINO ACID SEQUENCE OF CLONED S. PYOGENES DNase

R-Q-T-Q-V-S-N-D-V-V-L-N-D-G-A-S-K-Y-L-N-
E-A-L-A-W-T-F-N-D-S-P-N-Y-Y-K-T-L-G-T-S-
Q-I-T-P-A-L-F-P-K-A-G-D-I-L-Y-S-K-L-D-E-
L-G-R-T-R-T-A-R-G-T-L-T-Y-A-N-V-E-G-S-Y-
G-V-R-Q-S-F-G-K-N-Q-N-P-A-G-W-T-G-N-P-N-
H-V-K-Y-K-I-E-W-L-N-G-L-S-Y-V-G-D-F-W-N-
R-S-H-L-I-A-D-S-L-G-G-D-A-L-R-V-N-A-V-T-
G-T-R-T-Q-N-V-G-G-R-D-Q-K-G-G-M-R-Y-T-E-
Q-R-A-Q-E-W-L-E-A-N-R-D-G-Y-L-Y-Y-E-V-A-
P-I-Y-N-A-D-E-L-I-P-R-A-V-V-V-S-M-Q-S-S-
D-N-T-I-N-E-K-V-L-V-Y-N-T-A-N-G-Y-T-I-N-
Y-H-N-G-T-P-T-Q-K-

FIG. 4

PCR OLIGONUCLEOTIDE

5' TAACGGATCCGAATCTACTTGGATCAAGACGGGTTTTTTCT 3' (SEQ. ID NO: 2)

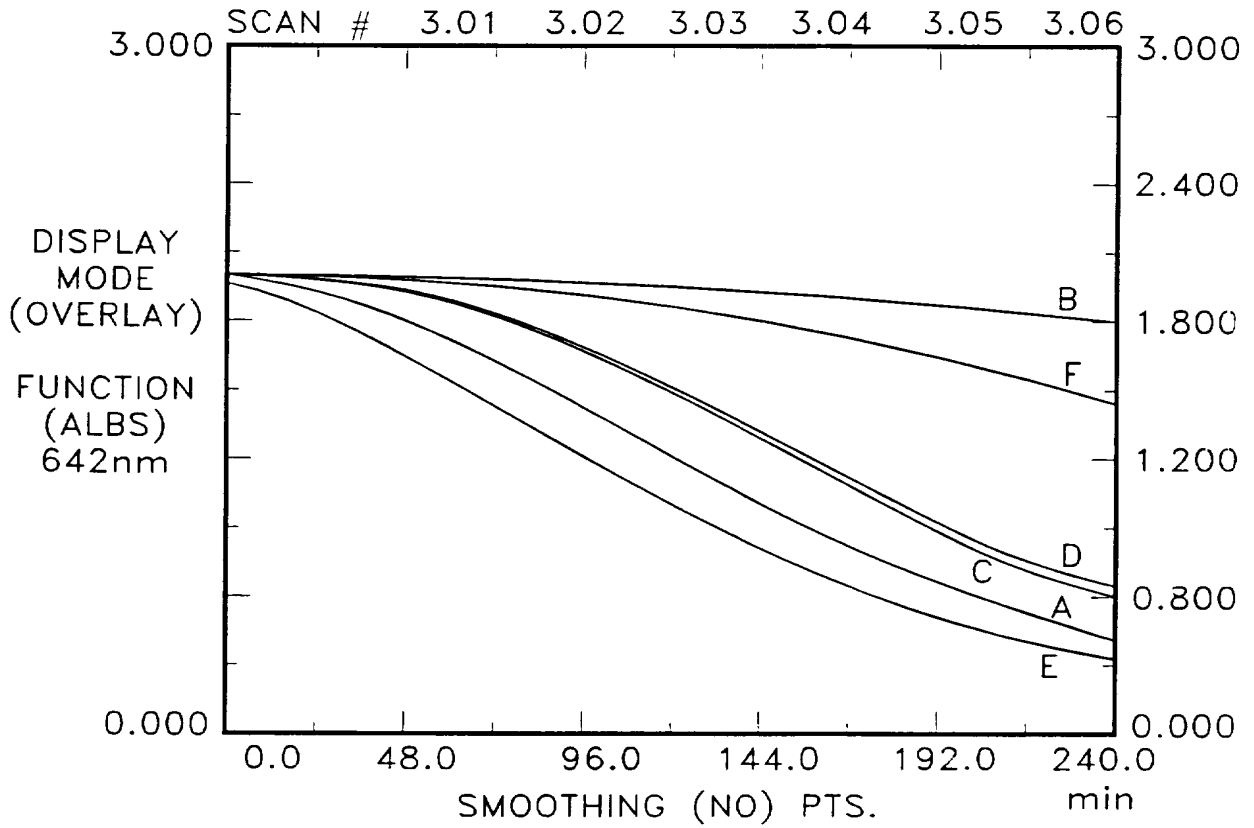
1 ATGGATCCGAATCTACTTGGATCAAGACGGGTTTTTTCTAAAAATCTCGGCTAGTAAA
 60 TACCTAGGCTTAGATGAACCTAGTTCTGCCCAAAAAGATTTTTTACAGCCGATCATTTT
 MetAspProAsnLeuLeuGlySerArgArgValPheSerLysLysCysArgLeuValLys
 61 TTTTCAATGGTAGCTCTTGTATCAGCCACAATGGCTGTAACAACAGTCACACTTGAAAAT
 120 AAAAGTTACCATCGAGAACATAGTCGGTGTACCGACATTGTTGTCAGTGTGAACTTTTA
 PheSerMetValAlaLeuValSerAlaThrMetAlaValThrThrValThrLeuGluAsn
 121 ACTGCACTGGCAGCACAACACAGGTCTCAAATGATGTTGTTCTAAATGATGGCGCAAGC
 180 TGACGTGACCGTGCTGTTTGTGTCCAGAGTTTACTACAACAAGATTTACTACCGCGTTTCG
 ThrAlaLeuAlaArgGlnThrGlnValSerAsnAspValValLeuAsnAspGlyAlaSer
 181 AAGTACCTAAACGAAGCATTAGCTTGGACATTC AATGACAGTCCTAACTATTACAAA
 240 TTCATGGATTTGCTTCGTAATCGAACCTGTAAGTTACTGTCAGGATTGATAATGTTTTGA
 LysTyrLeuAsnGluAlaLeuAlaTrpThrPheAsnAspSerProAsnTyrTyrLysThr
 241 TTAGGTACTAGTCAGATTACTCCAGCACTCTTTCCTAAAGCAGGAGATATTCTCTATAGC
 300 AATCCATGATCAGTCTAATGAGGTCGTGAGAAAGGATTTTCGTCCTCTATAAGAGATATCG
 LeuGlyThrSerGlnIleThrProAlaLeuPheProLysAlaGlyAspIleLeuTyrSer
 301 AAATTAGATGAGTTAGGAAGGACGCGTACTGCTAGAGGTACATTGACTTATGCCAATGTT
 360 TTTAATCTACTCAATCCTTCCTGCGCATGACGATCTCCATGTAACCTGAATACGGTTACAA
 LysLeuAspGluLeuGlyArgThrArgThrAlaArgGlyThrLeuThrTyrAlaAsnVal
 361 GAAGGTAGCTACGGTGTAGACAATCTTTCGGTAAAAATCAAAACCCCGCAGGATGGACT
 420 CTTCATCGATGCCACAATCTGTTAGAAAGCCATTTTTAGTTTTGGGGCGTCCTACCTGA
 GluGlySerTyrGlyValArgGlnSerPheGlyLysAsnGlnAsnProAlaGlyTrpThr
 421 GGAAACCCTAATCATGTCAAATATAAAATTGAATGGTTAAATGGTCTATCTTATGTCGGA
 480 CCTTTGGGATTAGTACAGTTTATATTTTAACTTACCAATTTACCAATAGAAATACAGCCT
 GlyAsnProAsnHisValLysTyrLysIleGluTrpLeuAsnGlyLeuSerTyrValGly

FIG. 5

481 GATTTCTGGAATAGAAGTCATCTCATTGCAGATAGTCTCGGTGGAGATGCACTCAGAGTC 540
 -----+-----+-----+-----+-----+-----+-----+
 CTAAGACCTTATCTTCAGTAGAGTAACGTCTATCAGAGCCACCTCTACGTGAGTCTCAG
 AspPheTrpAsnArgSerHisLeuIleAlaAspSerLeuGlyGlyAspAlaLeuArgVal
 541 AATGCCGTTACAGGAACACGTACCCAAAATGTAGGAGGTCGTGACCAAAAAGGCGGCATG 600
 -----+-----+-----+-----+-----+-----+-----+
 TTACGGCAATGTCCTTGTGCATGGGTTTTACATCCTCCAGCACTGGTTTTTCCGCCGTAC
 AsnAlaValThrGlyThrArgThrGlnAsnValGlyGlyArgAspGlnLysGlyGlyMet
 601 CGCTATACCGAACAAAGAGCTCAAGAATGGTTAGAAGCAAATCGTGATGGCTATCTTTAT 660
 -----+-----+-----+-----+-----+-----+-----+
 GCGATATGGCTTGTCTCGAGTTCTTACCAATCTTCGTTTAGCACTACCGATAGAAATA
 ArgTyrThrGluGlnArgAlaGlnGluTrpLeuGluAlaAsnArgAspGlyTyrLeuTyr
 661 TATGAAGTCGCTCCAATCTACAACGCAGACGAGTTGATTCCAAGAGCTGTCTGGTATCA 720
 -----+-----+-----+-----+-----+-----+-----+
 AACTTCAGCGAGGTTAGATGTTGCGTCTGGTCAACTAAGGTTCTCCACAGCACCATAGT
 TyrGluValAlaProIleTyrAsnAlaAspGluLeuIleProArgAlaValValValSer
 721 ATGCAATCTTCTGATAATACCATCAACGAGAAAGTATTAGTTTAGAACAGAGCTAATGGC 780
 -----+-----+-----+-----+-----+-----+-----+
 TACGTTAGAAGACTATTATGGTAGTTGCTCTTTCATAATCAAATGTTGTGTGCGATTACCG
 MetGlnSerSerAspAsnThrIleAsnGluLysValLeuValTyrAsnThrAlaAsnGly
 781 TACACCATTAACTACCATAACGGTACACCTACTCAAAAATAATACCAAAAGGCTAGACCT 840
 -----+-----+-----+-----+-----+-----+-----+
 ATGTGGTAATTGATGGTATTGCCATGTGGATGAGTTTTTATTATGGTTTTCCGATCTGGA
 TyrThrIleAsnTyrHisAsnGlyThrProThrGlnLysEndTyrGlnLysAlaArgPro
 841 CTGCTCACTAGGCCTAGCTTTTTTACATCAAAAAAGCAATGACTATAGAAAGTAAAAATA 900
 -----+-----+-----+-----+-----+-----+-----+
 GACGAGTGATCCGGATCGAAAAATGTAGTTTTTTTCGTTACTGATATCTTTCATTTTTAT
 LeuLeuThrArgProSerPheLeuHisGlnLysLysGlnEnd
 901 CTAGAAAAAGCAATGATTGCCGTCATTGCCCGGGTCGACCCGG 944 (SEQ. ID NO: 1)
 -----+-----+-----+-----+-----+-----+-----+
 GATCTTTTTTCGTTACTAACGGCAGTAACGGGGCCCAGCTGGGCC
 3' TCTTTTTTCGTTACTAACGGCAGTAACGGGGCCCAGCTGGGCC 5' (SEQ. ID NO: 3)

PCR OLIGONUCLEOTIDE

FIG. 5 (cont.)



A=STREPTONASE B
 B=STREPTONASE B + ANTIBODY
 C=DNase1-1
 D=DNase1-1 + ANTIBODY
 E=DNase B2-6
 F=DNase B2-6 + ANTIBODY

FIG. 6

CORRELATION CURVE OF ACTIVITY ASSAYS
BASED ON RECOMBINANT AND NATURAL
(STREPTONASE B) DNAase B

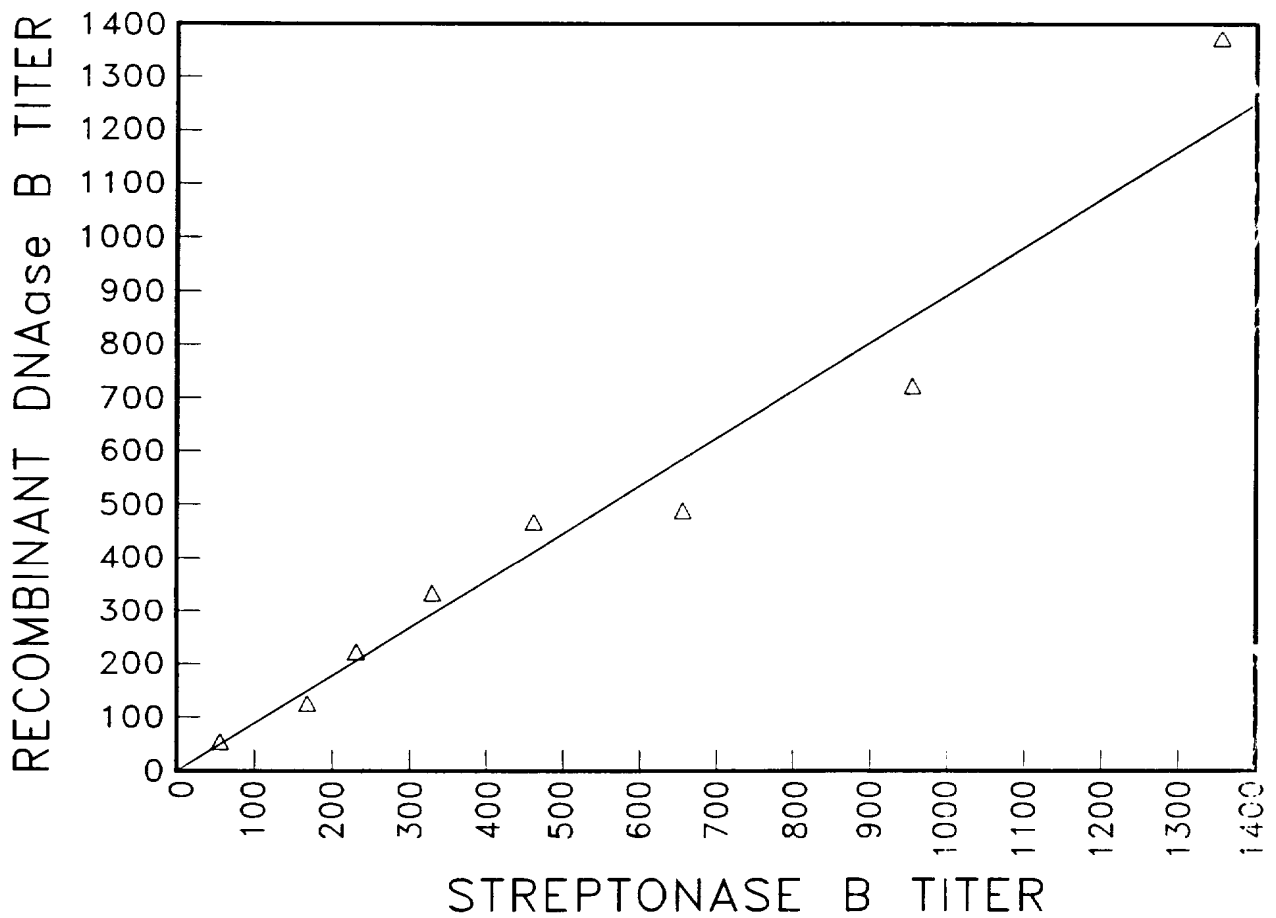


FIG. 8

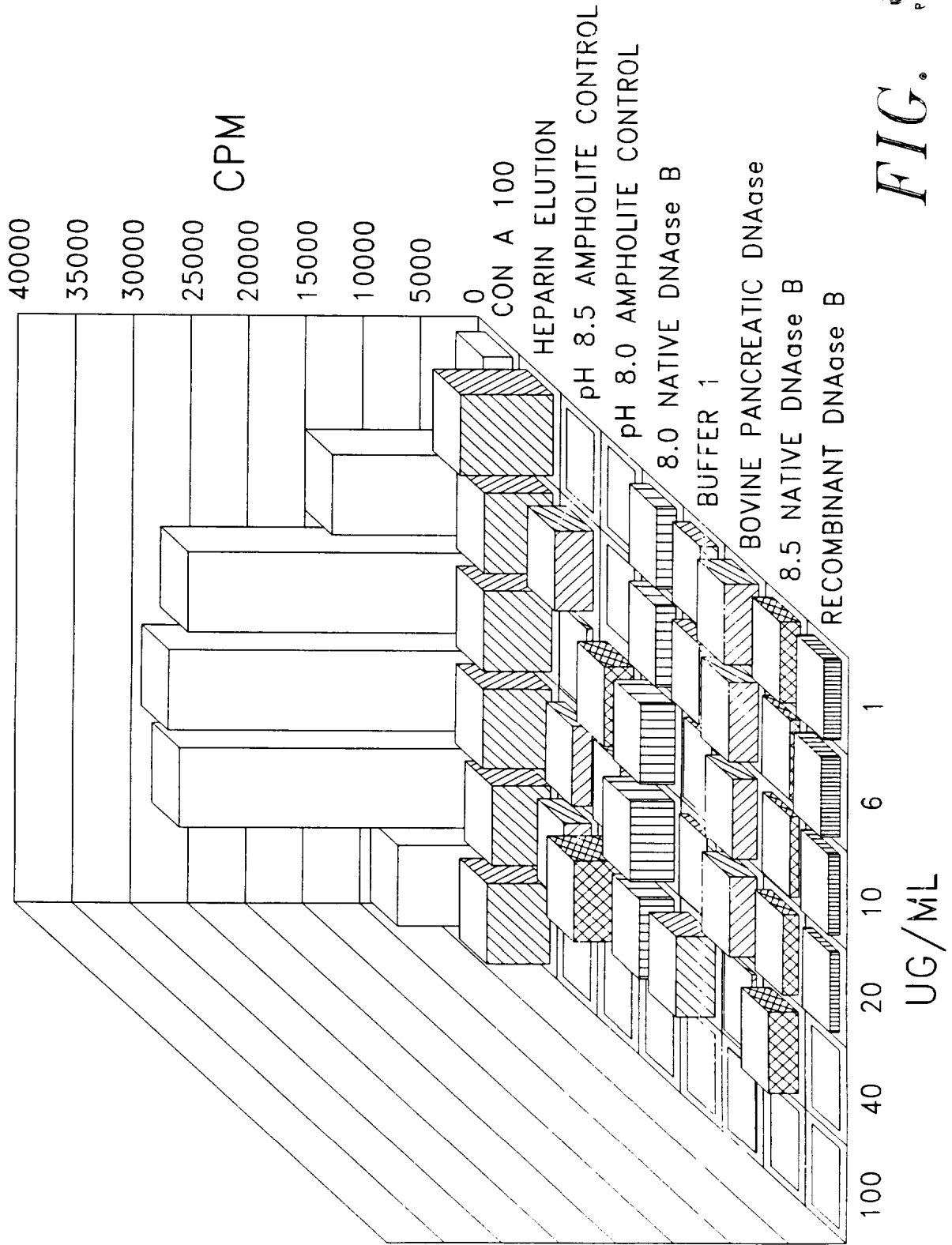


FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/09450

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 6	C12N15/55	C12N15/70	C12N15/62	C12N1/21	C12N9/22
	C07K1/36	C12Q1/68	C12P21/08	G01N33/573	

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C07K C12Q C12P G01N
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>FEBS LETTERS., vol. 308, ELSEVIER, AMSTERDAM, NL, pages 30-34, TAKASHI YUTSUDO ET AL. 'A new type of mitogenic factor produced by Streptococcus pyogenes' cited in the application see abstract see page 30, right column, last paragraph - page 31, left column, paragraph 3 see page 32, left column, last paragraph - page 33, left column, paragraph 1 see page 33, left column, paragraph 3 - right column, paragraph 1</p> <p align="center">--- -/--</p>	10, 25

Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

A document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
E earlier document but published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
O document referring to an oral disclosure, use, exhibition or other means	* & * document member of the same patent family
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 3 April 1995	Date of mailing of the international search report 11 -04- 1995
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Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Authorized officer Hornig, H
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/09450

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FEBS LETTERS., vol. 331,no. 1,2, 27 September 1993 AMSTERDAM NL, pages 187-192, MAKOTO IWASAKI ET AL. 'Cloning, characterization and overexpression of a Streptococcus pyogenes gene encoding a new type of mitogenic factor'	1,2,4,8, 10,11, 13-15, 18,19, 27,28
Y	see abstract see page 187, left column, paragraph 2 - paragraph 3 see page 188, left column, paragraph 2 - paragraph 3 see page 188, right column, paragraph 3 - page 189, right column, paragraph 1; figure 3 see page 191, left column, paragraph 1 see page 192, left column, paragraph 2 ---	35,36, 39,40
Y	THE JOURNAL OF LABORATORY AND CLIN. MEDICINE, vol. 95,no. 2, February 1980 C.V.MOSBY,ST. LOUIS,US;, pages 258-265, MICHAEL A. GERBER ET AL. 'Enzyme-linked immunosorbent assay of antibodies in human sera to streptococcal DNase B' cited in the application see page 259, paragraph 2 see page 259, paragraph 5 see page 259, paragraph 8 - page 260, paragraph 1 ---	39,40
Y	EP,A,0 266 686 (BEHRINGWERKE) 11 May 1988 cited in the application see page 2, line 46 - line 52; claim 1 ---	35,36
E	EP,A,0 613 947 (SHIONOGI CO., LTD.) 7 September 1994 see page 2, line 36 - line 48 see page 2, line 57 - line 58 see page 6, line 46 - line 58 see page 3, line 5 - page 5, line 5 see page 5, line 55 - page 6, line 7 ---	1,2,4,8, 10,11, 13-15, 27,28, 31,32
2 E	WO,A,95 00650 (BECKMAN INSTRUMENTS, INC.) 5 January 1995 see page 32, line 19 - line 24 see page 12, line 28 - page 32, line 16; claims 1-54; examples 1-11 -----	1-63

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/09450

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 60-63 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No
PCT/US 94/09450

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